Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Animal

Clams from Tagus estuary: microbiological, physiological and chemical responses to depuration, transport and environmental stress

Patrícia Sofia Laranjeira Anacleto

Doutoramento em Biologia

Especialidade em Biologia Marinha e Aquacultura

2014

Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Animal

Clams from Tagus estuary: microbiological, physiological and chemical responses to depuration, transport and environmental stress

Patrícia Sofia Laranjeira Anacleto

Tese orientada pelo Investigador Principal Doutor Rui Afonso Bairrão da Rosa e pelo Investigador Auxiliar Doutor António Manuel Barros Marques, especialmente elaborada para a obtenção do grau de Doutor em Biologia
Especialidade em Biologia Marinha e Aquacultura

2014

Doctoral dissertation in Biology

(specialization scientific area in Marine Biology and Aquaculture) presented to the University of Lisboa

Dissertação apresentada à Universidade de Lisboa para obtenção do grau de Doutor

(especialidade Biologia Marinha e Aquacultura)

Patrícia Sofia Laranjeira Anacleto

2014

AGRADECIMENTOS

Durante a realização desta tese de doutoramento, várias pessoas estiveram envolvidas, directa ou indirectamente, no trabalho e a quem quero expressar os meus sinceros e profundos agradecimentos.

Em primeiro lugar, agradeço especialmente aos meus orientadores que foram excepcionais e são um exemplo a seguir:

- Doutor António Marques, que sempre me incentivou a realizar este doutoramento e me deu suporte para que tal fosse possível. Além de sempre me motivar e apoiar incansavelmente, esteve sempre presente em todas as fases deste trabalho científico, desde os longos embarques no rio Tejo até às noitadas no laboratório com as amêijoas. Além de um grande amigo, ajudou-me a ultrapassar todos os obstáculos impostos e a conseguir superar todas as dificuldades que encontramos na investigação. Agradeço também todos os conhecimentos científicos e experiência que me transmitiu diariamente e por toda a disponibilidade constante sempre demonstrada. Quero ressaltar também toda a sua paciência em me "aturar" e todo o seu positivismo transmitido que foram uma mais-valia e vontade de continuar em frente.

- Doutor Rui Rosa, que me aceitou como sua doutoranda e me deu esta oportunidade única de trabalhar também noutra área diferente e tão interessante da habitual ("Food"). É um privilégio tê-lo como orientador e trabalhar num local tão magnífico como é a "Guia". A minha profunda gratidão pela amizade, simpatia, motivação, confiança, empenho, entusiasmo e inspiração em todas as etapas deste trabalho científico. Agradeço também todos os conhecimentos científicos transmitidos e financeiros disponibilizados. É extraordinário trabalhar com uma pessoa que tem uma visão científica tão abrangente, com ideias inovadoras constantes e com um espírito incondicional para o mundo científico.

Agradeço à Doutora Maria Leonor Nunes pelo seu apoio, orientação e supervisão atenta nas várias fases deste trabalho. Tem sido sempre um privilégio poder contar com a sua dedicação, inspiração, sabedoria, empenho, confiança e motivação. Além disso, agradeço prontamente todas as suas sugestões, críticas e esclarecimento de dúvidas no decorrer deste trabalho científico.

Agradeço às instituições de acolhimento, o Instituto Português do Mar e da Atmosfera (IPMA, I.P.) e ao Laboratório Marítimo da Guia (Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa; LMG-CO, FCUL, UL), por terem proporcionado as condições necessárias à realização deste trabalho científico.

Agradeço a todas as pessoas da Divisão de Aquacultura e Valorização (DivAV) do IPMA que estiveram envolvidas neste trabalho científico. Em especial à Dra. Ana Luísa Maulvault pela amizade dentro e fora do meio científico, pelo apoio e ajuda sempre demonstrados no trabalho prático, em particular quando incluía fazer noitadas e passar fins-de-semana com as amêijoas, muitas vezes com um odor insuportável. Toda a sua boa-disposição e energia constantes foram uma mais-valia para que esses momentos fossem menos duros.

Agradeço também o apoio prestado e amizade demonstrada da Doutora Milena Chaguri e da Doutora Paula Martin, durante as suas estadias em Portugal e que certamente levaram boas recordações para o Brasil. Assim como eu trouxe do Brasil e agradeço à Milena pela grande amizade e pelos passeios maravilhosos por Botucatu. Agradeço à Doutora Narcisa Bandarra, Doutor Rogério Mendes e Doutora Sónia Pedro pela colaboração no trabalho desenvolvido nos laboratórios de Nutrição, Bioquímica e Microbiologia. Muitos outros colegas foram fundamentais no apoio dado, entre os quais o Doutor Carlos Cardoso, Doutora Sara Barrento, Júlia Ferreira, Engª Patrícia Oliveira, Doutora Helena Silva, Doutora Helena Lourenço, Doutora Cláudia Afonso e Dra. Vera Barbosa. E agradeçço a todos os restantes colegas…

Do Laboratório Marítimo da Guia, agradeço a todos os colegas pelo companheirismo e disponibilidade demonstrados. Em especial à Dra. Vanessa Lopes, Doutor Tiago Repolho, Dr. Miguel Baptista e Dra. Ana Rita Lopes pelo apoio constante que deram durante as experiências, pela vossa amizade e por serem simplesmente espectaculares.

Agradeço ao Doutor Mário Diniz por me ter gentilmente recebido na Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa (FCT/UNL) e por todos os seus conhecimentos científicos e colaboração no trabalho aí desenvolvido. Igualmente, quero agradecer à Dra. Diana Madeira e Dra. Vanessa Mendonça pela ajuda e apoio na realização das análises laboratoriais.

Quero agradecer ao Doutor Patrick Freire do Instituto Nacional de Investigação Agrária e Veterinária (INIAV, I.P.) pela sua disponibilidade, ensinamentos, apoio e boa-disposição sempre demonstradas. Quero também agradecer à Dra. Tânia Chança pelo grande apoio e ajuda nas últimas experiências com as amêijoas e desejar boa sorte para a conclusão do seu mestrado. À Doutora Inês Guinote pela simpatia e bons momentos de convívio, principalmente nas horas de almoço.

Agradeço à Professora Doutora Maria Luísa Carvalho do laboratório do Centro de Física Atómica da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa (CFA, FCT/UNL) assim como a todas as meninas da sua equipa de investigação (Sofia, Ana, Diana, Marta) pela colaboração, apoio e ajuda na determinação de elementos químicos no aparelho de EDXRF.

Agradeço a todos os pescadores que colaboraram gentilmente e com entusiasmo na recolha de amostras no estuário do Tejo, em particular ao Sr. Jorge Camarão Jerónimo, Sr. Sandro Pinto, Sr. Pedro Patacas e Sr. Daniel.

Agradeço ao Aquário Vasco da Gama, e em especial à Dra. Fátima Gil e Dra. Sara Chalante por serem tão amáveis e cederem microalgas sempre que necessário.

Agradeço à Fundação para a Ciência e Tecnologia (FCT) pelo financiamento da minha bolsa de doutoramento (Ref: SFRH/BD/69274/2010) que possibilitou a execução desta tese e também aos projectos MUSSELSALIVE “Development of best practice and new technology for grading, handling, transportation, conditioning and storage of mussels for SMEs in the European mussel industry” (Ref: 243452) e SHELLPLANT “Development of a novel production system for intensive and cost effective bivalve farming” (Ref: 232273) que financiaram muitos dos reagentes e material.

Agradeço a todos os meus amigos, pelo seu apoio, amizade, motivação e pelos bons momentos partilhados.

Agradeço à minha família, e de um modo muito especial aos meus pais pelo amor incondicional, educação, carinho, apoio e incentivo em todos os momentos da minha vida. Obrigado por sempre me proporcionarem as melhores condições e serem o meu grande suporte. Agradeço especialmente à minha irmã Andreia por todo o apoio incondicional, pela grande ajuda que me deu nos trabalhos com as amêijoas, inclusive quando consistia em "lavar sistemas" e por toda a motivação, amizade e carinho que foram constantes e fundamentais durante estes anos. Agradeço também toda a ajuda e força do João Pedro que foram muito importantes e fundamentais.

Por último, mas não menos importante e decerto o primeiro, agradeço ao Nuno, pelo seu amor, amizade, carinho, companheirismo, incentivo, compreensão, paciência, apoio incondicional e motivação constantes, o que sempre me transmitiu segurança e vontade de concluir esta tese. Obrigada por tudo o que fizeste por mim e continuas a fazer. Sei que posso contar sempre contigo. Quero também agradecer ao Lucky, o meu gatinho "mais que tudo", pelos seus miminhos e carinhos que recebi aquando da escrita da tese. De igual modo, agradecimentos felinos ao Savi, à Sasha e ao Suri.

Obrigado a todos aqueles que de alguma forma contribuíram e participaram em mais uma etapa da minha vida.

TABLE OF CONTENTS

List of abbreviations and units 1

List of figures 9

List of tables 13

Abstract 17

Resumo 19

Resumo alargado 21

List of papers 25

CHAPTER 1 – General introduction 27

CHAPTER 2 – Portuguese consumers' attitudes and perceptions of bivalve molluscs 77

CHAPTER 3 – Microbiological composition of native and exotic clams from Tagus estuary: Effect of season and environmental parameters 89

CHAPTER 4 – Microbiological responses to depuration and transport of native and exotic clams at optimal and stressful temperatures 101

CHAPTER 5 – Physiological responses to depuration and transport of native and exotic clams at different temperatures 113

CHAPTER 6 – Effects of depuration on metal levels and health status of bivalve molluscs 127

CHAPTER 7 – Ecophysiology of native and alien invasive clams in an ocean warming context 145

CHAPTER 8 – Effect of warming on nutritional quality of native and alien invasive clams 159

CHAPTER 9 – General discussion and conclusions 173

ANNEX 199

LIST OF UNITS AND ABBREVIATIONS

%	percentage
/	per
€	euros
±	approximately
°C	degree Celsius
atm	atmosphere
cm	centimetre(s)
dw	dry weight
eV	electronvolt
g	gram(s)
g	relative centrifugal force or G-force
h	hour(s)
Kcal	kilocalories
kDa	kilodalton
keV	kiloelectronvolt
kg	kilogram(s)
kJ	kilojoule(s)
km^2	square kilometre
kPa	kilopascal
kV	kilovolts
L	litre(s)
log	logarithm
m	metre(s)
M	molar concentration
mA	milliampere(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mM	milimolar
mm	millimetre(s)
mt	metric tonne(s)
ng	nanogram(s)
nm	nanometer(s)

nmol	nanomole
PFU	plaque forming units
pg	picogram(s)
ppt	part per thousand
PSU	practical salinity units
rpm	revolutions per minute
s or sec	second(s)
t	tonne(s)
U	units
V	volt(s)
v/v	volume per volume
W	watt(s)
w/v	weight per volume
ww	wet weight
γ	gamma
λ	wavelength
µg	microgramа(s)
µL	microlitre(s)
µm	micrometre(s)
µM	micromolar
µmol	micromole
χ^2	chi-square test
ω	omega
AA	arachidonic acid
ADP	adenosine 5'-diphosphate
AEC	adenylate energy charge
AI	atherogenicity index
AIS	alien invasive species
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
APW	alkaline peptone water
As	arsenic
ASP	amnesic shellfish poisoning
ATP	adenosine 5'-triphosphate
BGA	brilliant green agar
BPW	buffered peptone water

Br	bromine
BSA	bovine serum albumin
Ca	calcium
CAT	catalase
Cd	cadmium
CDNB	l-chloro-2,4-dinitrobenzene
CFU	colony-forming units
CI	condition index
Cl	chlorine
Co	cobalt
CO_2	carbon dioxide
Cr	chromium
CS	citrate synthase
CTMax	critical thermal maximum
CTMin	critical thermal minimum
Cu	copper
D.O.	dissolved oxygen
DDT	dichloro-diphenyl-trichloro-ethane
DGPA	Direcção Geral das Pescas e Aquicultura
DHA	docosahexaenoic acid
DL	detection limits
DNA	deoxyribonucleic acid
DR	Diário da República
DSP	diarrhetic shellfish poisoning
DT22	depurated and transported clams at 22 °C
DT4	depurated and transported clams at 4 °C
e.g.	for example
EA	erucic acid
EC	European Commission
EDTA	ethylenediaminetetraacetic acid
EDXRF	energy dispersive X-ray fluorescence
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
etc.	*Et cetera*
EU	European Union
FA	fatty acids

FAAS	flame atomic-absorption spectrometry
FAME	fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FC	faecal coliforms
FDA	Food and Drug Administration
Fe	iron
GA	gadoleic acid
GISP	Global Invasive Species Programme
GPX	glutathione peroxidise
GR	glutathione reductase
GSH	reduced glutathione
GST	glutathione S-transferase
h/H	hypocholesterolemic/hypercholesterolemic indice
H_2O	water
H_2O_2	hydrogen peroxide
HAB	harmful algal blooms
HAV	hepatitis A virus
Hg	mercury
HO	hydroxyl radical
HPLC	high performance liquid chromatography
HPP	high pressure processing
HSC70	heat shock cognate 70
HSP24	heat shock protein 24
HSP70	heat shock protein 70
HSR	heat shock response
HTA	hexadecatrienoic acid
HVA	homeoviscous adaptation
Hx	hypoxanthine
HxR	inosine
i.e.	*is est*/that is/in other words
ICMSF	International Commission on Microbiological Specifications for Foods
IMP	inosine 5'-monophosphate
INE	National Institute of Statistics
IPCC	Intergovernmental Panel on Climate Change
IPMA	Portuguese Institute for the Sea and Atmosphere
IS	internal standard

ISO	International Organization for Standardization
K	potassium
LA	linoleic acid
LL	legal limit
LT100	temperature required for 100% of mortality
LT50	time/temperature required for 50% mortality
MDA	malondialdehyde
MDH	malate dehydrogenase
meHg	methylmercury
Mg	magnesium
MG	*Mytilus galloprovincialis*
Mn	manganese
MPLs	maximum permissible limits
MPN	most probable number
MRD	maximum recovery diluent
MUFA	monounsaturated fatty acids
n or N	number of replicates
n.a.	not analysed
n-3	omega-3 fatty acids
n-6	omega-6 fatty acids
Na	sodium
NBT	nitroblue tetrazolium
ND	not detected
NE	not eliminated
Ni	nickel
NLV	Norwalk-like virus
NOAA	National Oceanic and Atmospheric Administration
NSP	neurotoxic shellfish poisoning
O_2	oxygen
OA	oleic acid
p or *p*-value	probability of the test statistic
PA	palmitic acid
PAHs	polycyclic aromatic hydrocarbons
Pb	lead
PBS	phosphate-buffered saline solution
PBST	phosphate-buffered saline solution Tween-20

PCBs	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-p-dioxins
PCDF	polychlorinated dibenzofurans
P_{crit}	critical oxygen partial pressure
pH	measure of the acidity or basicity of an aqueous solution
PO_2	oxygen partial pressure
POPs	persistent organic pollutants
PSP	paralytic shellfish poisoning
PUFA	polyunsaturated fatty acids
Q_{10}	thermal sensitivity
QMRA	quantitative microbial risk assessment
r	correlation coefficient
RAS	recirculation aquaculture system
RASFF	Rapid Alert System for Food and Feed
Rb	rubidium
RL	recommended limit
RMRs	routine metabolic rates
ROS	reactive oxygen species
RP	*Ruditapes philippinarum*
S	sulphur
SA	stearic acid
SARF	Scottish Aquaculture Research Forum
SD	standard deviation
SDS	sodium dodecyl sulfate
Se	selenium
SFA	saturated fatty acids
SNIRH	National Information System of Water Resources
SOD	superoxide dismutase
SP	*Scrobicularia plana*
sp. or spp.	specific epithet of species not identified. Single or several species within a genus
Sr	strontium
SRTEM	*Salmonella* rapid test medium
T22	non-depurated clams and transported at 22 °C
T4	non-depurated clams and transported at 4 °C
TBARS	thiobarbituric acid reactive substances

TBT	tributyltin
TBX	tryptone bile X-glucuronide agar
TCBS	thiosulphate citrate bile salt sucrose agar
TI	thrombogenicity index
T_{off}	cessation temperature
T_{on}	onset temperature
T_{peak}	temperature of maximal induction
TPHs	total petroleum hydrocarbons
TSA	trypticase soy agar
TSI	triple sugar iron agar
TVC	total viable counts
UK	United Kingdom
UL	undetected limits
USA	United States of America
USEPA	United States Environmental Protection Agency
UV	ultraviolet light
UV-Vis	ultraviolet–visible
VA	vaccenic acid
VP	*Venerupis pullastra*
WB	western blot
WHO	World Health Organization
WTTC	World Travel & Tourism Council
XLD	xylose–lysine–desoxycholate agar
XOD	xanthine oxidase solution
Zn	zinc

LIST OF FIGURES

CHAPTER 1 – General introduction

Fig. 1. World production (fisheries and aquaculture) between 1950 and 2011 in tonnes (t) of the most important groups of seafood (a) and the most representative bivalve groups (b).

Fig. 2. World production (fisheries and aquaculture) between 1950 and 2011 in tonnes (t) of main produced bivalves' species.

Fig. 3. Major bivalve producing countries (fisheries and aquaculture) of different bivalve mollusc groups, data in tonnes (t) from 1950 to 2011.

Fig. 4. National production (fisheries and aquaculture) in tonnes (t) of the most representative bivalves from the Portuguese coast between 1950 and 2012.

Fig. 5. Imports and exports of bivalves by Portugal, data in value (€ million) from 1988 to 2013.

Fig. 6. Main importers of bivalves from Portugal, data in tonnes (t) from 1988 to 2013.

Fig. 7. Main exporters of bivalves to Portugal, data in tonnes (t) from 1988 to 2013.

Fig. 8. National trade chain of live bivalve molluscs from harvest to consumers.

Fig. 9. Hypothetical physiological performance curves for ectothermic animals.

Fig. 10. Conceptual model of greater invasive eurythermality.

CHAPTER 2 – Portuguese consumers' attitudes and perceptions of bivalve molluscs

Fig. 1. Preferred bivalve species in Portugal (n = 1419).

CHAPTER 3 – Microbiological composition of native and exotic clams from Tagus estuary: Effect of season and environmental parameters

Fig. 1. Map showing Tagus estuary (Portugal) and sampling locations (Trafaria for *V. pullastra* and Barreiro for *R. philippinarum*).

Fig. 2. Seasonal variations of *Escherichia coli* levels in (A) *Venerupis pullastra,* and (B) *Ruditapes philippinarum* clams, sediment (log CFU/100 g) and harvesting waters (log CFU/100 mL) from Trafaria and Barreiro.

Fig. 3. Seasonal variations of TVC levels in (A) *Venerupis pullastra* and (B) *Ruditapes philippinarum* clams, sediment (log CFU/g) and harvesting waters (log CFU/mL) from Trafaria and Barreiro.

Fig. 4. Seasonal variations of *Vibrio* spp. levels in (A) *Venerupis pullastra* and (B) *Ruditapes philippinarum*, sediment (log CFU/g) and harvesting waters (log CFU/mL) from Trafaria and Barreiro.

CHAPTER 4 – Microbiological responses to depuration and transport of native and exotic clams at optimal and stressful temperatures

Fig. 1. Map of Portugal with the enlarged portion of Tagus estuary. Dark filled circles indicate the samplings sites (Trafaria for *V. pullastra* and Alcochete for *R. philippinarum*).

Fig. 2. Cumulative mortality rate (%) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry simulated transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry simulated transport at 4 or 22 °C.

Fig. 3. TVC levels (log CFU/g) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry simulated transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry simulated transport at 4 or 22 °C.

Fig. 4. *Escherichia coli* levels (CFU/100 g) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semidry simulated transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry simulated transport at 4 or 22 °C.

Fig. 5. *Vibrio* spp. levels (log CFU/g) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry simulated transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry simulated transport at 4 or 22 °C.

CHAPTER 5 – Physiological responses to depuration and transport of native and exotic clams at different temperatures

Fig. 1. Map of Portugal with enlarged portion of the Tagus estuary. Dark dots indicate the samplings sites (Trafaria for *V. pullastra* and Alcochete for *R. philippinarum*).

Fig. 2. Schematic representation of the experimental design.

Fig. 3. Survival rate (%) of native (*Venerupis pullastra*, VP) and exotic (*Ruditapes philippinarum*, RP) clams subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C.

Fig. 4. Condition index (CI) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C.

Fig. 5. Changes in levels of ATP-related compounds (μmol/g, wet weight) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to depuration followed by transported after at 4 or 22 °C.

Fig. 6. Changes in levels of ATP-related compounds (μmol/g, wet weight) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to semi-dry transport at 4 or 22 °C without depuration.

Fig. 7. K-value (%) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C.

Fig. 8. A.E.C. value (%) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C.

Fig. 9. Glycogen content (g/100 g, wet weight) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C.

CHAPTER 6 – Effects of depuration on metal levels and health status of bivalve molluscs

Fig. 1. Map of Portugal with enlarged portion of the Tagus estuary. Dark dots indicate the samplings sites (Alcochete for RP, Algés for MG and Sítio das Hortas for SP).

Fig. 2. Glycogen content (g/100 g, wet weight) of RP, MG and SP during the depuration time.

Fig. 3. Toxic elements concentration (Hg, Cd, Pb and As; mg/kg wet weight) of RP, MG and SP during depuration time. Values represent mean ± standard deviation (n = 3).

CHAPTER 7 – Ecophysiology of native and alien invasive clams in an ocean warming context

Fig. 1. Effect of environmental warming on the cumulative mortality rates (%) of native (*Ruditapes decussatus*) and alien invasive (*Ruditapes philippinarum*) clams.

Fig. 2. Effect of environmental warming on the thermal tolerance limits (CTMax, °C) of native (*Ruditapes decussatus*, n=60) and alien invasive (*Ruditapes philippinarum*, n=60) clams.

Fig. 3. Effect of environmental warming on the routine metabolic rates (RMRs, μmol O_2/g/h wet weight) of: A) native (*Ruditapes decussatus*) and B) alien invasive (*Ruditapes philippinarum*) clams.

Fig. 4. Effect of environmental warming on thermal sensitivity (Q_{10} values) of: A) native (*Ruditapes decussatus*) and B) alien invasive (*Ruditapes philippinarum*) clams.

Fig. 5. Effect of environmental warming on the valve closure behaviour (as % of open and closed valves) of: A) native (*Ruditapes decussatus*) and B) alien invasive (*Ruditapes philippinarum*) clams (n=5 per each temperature).

Fig. 6. Effect of environmental warming on the routine metabolic rates (RMR, μmol O_2/g/h wet weight) as a function of oxygen partial pressure (PO_2; KPa) in: A) native (*Ruditapes decussatus*) and B) alien invasive (*Ruditapes philippinarum*) clams.

Fig. 7. Effect of environmental warming on: A) heat shock response (HSP70/HSC70, μg/μg total protein) and B) lipid peroxidation (expressed as MDA concentration, nmol/min/μg total protein) of native (*Ruditapes decussatus*) and alien invasive (*Ruditapes philippinarum*) clams.

Fig. 8. Representative western blot from both clam species exposed to 30 °C, showing differences in HSP70 levels between these species.

Fig. 9. Effect of environmental warming on the antioxidant enzyme activities of native (*Ruditapes decussatus*) and alien invasive (*Ruditapes philippinarum*) clams. A) glutathione-S-transferase (GST, nmol/min/μg total protein), B) catalase (CAT, nmol/min/μg total protein) and C) superoxide-dismutase (SOD, U/μg total protein).

Fig. S1. Map of the sampling area (red circles) in Tagus (*R. philippinarum*) and Sado (*R. decussatus*) estuaries.

CHAPTER 8 – **Effect of warming on protein, glycogen and fatty acid content of native and invasive clams**

Fig. 1. Effect of warming on A) protein and B) glycogen contents (mg/g dw) of the native clam (*R. decussatus*) and the invasive clam (*R. philippinarum*).

CHAPTER 9 – General discussion and conclusions

No figures

LIST OF TABLES

CHAPTER 1 – General introduction

Table 1. Biochemical composition of the most consumed bivalve molluscs in Portugal.

Table 2. Hazards associated with bivalve molluscs' consumption.

Table 3. Examples of *Escherichia coli* accumulation factors in bivalve species.

Table 4. European criteria for the classification of bivalve mollusc harvesting areas.

Table 5. Regulatory levels for toxins from harmful algal blooms (HAB) and chemical contaminants in live bivalve molluscs.

Table 6. Recommended temperature range, minimum salinity limit, permissible loading densities and maximum depth per tray for depuration of different bivalve species.

Table 7. Examples of the effect of depuration on the elimination of microbial and chemical contaminants from bivalves.

Table 8. Physiological responses of native and alien invasive species (AIS) to temperature increase.

CHAPTER 2 – Portuguese consumers' attitudes and perceptions of bivalve molluscs

Table 1. Demographic characteristics of survey respondents (n = 1778) and Portuguese population with internet access.

Table 2. Consumption of bivalves and reasons (%).

Table 3. Consumers' pattern of clams (%) and perceived knowledge of risks associated with its consumption (n = 1419).

Table 4. Consumers' quality criteria (%) for choosing different bivalve species.

Table 5. Consumer's quality criteria (%) for choosing clams (n = 1419).

Table 6. Consumers' attitude to pullet carpet shell clam (*Venerupis pullastra*) with respect to criteria of participants' demographics and socio-economic status (n = 370). Frequency of consumption and amount per meal (%).

Table 7. Consumers' attitude to Japanese carpet shell clam (*Ruditapes philippinarum*) with respect to criteria of participants' demographics and socio-economic status (n = 270). Frequency of consumption and amount per meal (%).

CHAPTER 3 – Microbiological composition of native and exotic clams from Tagus estuary: Effect of season and environmental parameters

Table 1. Seasonal variation of physico-chemical parameters of the water samples at the two sampling locations (Trafaria and Barreiro).

Table 2. Results of two-way ANOVA evaluating the effects of season and species (*Venerupis pullastra* and *Ruditapes philippinarum*) on TVC, *Escherichia coli* and *Vibrio* spp. levels.

Table 3. Occurrence of *Salmonella* spp. and *Vibrio* spp. in clams (*Venerupis pullastra*, VP, and *Ruditapes philippinarum*, RP), water and sediment from Trafaria and Barreiro over a one-year period.

Table 4. Pearson correlation coefficients between clams, water and sediment samples in each pathogenic bacteria studied.

Table 5. Pearson correlation coefficients between the environmental parameters and pathogenic bacteria levels.

CHAPTER 4 – Microbiological responses to depuration and transport of native and exotic clams at optimal and stressful temperatures

Table 1. Summary of criteria used for scoring behavioural clam activity.

Table 2. Results of two-way ANOVA evaluating the effects of simulated transport temperature (4 and 22 °C) and species (*Venerupis pullastra* and *Ruditapes philippinarum*) on cumulative mortality, TVC, *Escherichia coli* and *Vibrio* spp. levels.

Table 3. Pearson correlation coefficients between cumulative mortality, TVC, *Escherichia coli* and *Vibrio* spp. levels in each treatment of *Ruditapes philippinarum*.

Table 4. Pearson correlation coefficients between cumulative mortality, TVC, *Escherichia coli* and *Vibrio* spp. levels in each treatment of *Venerupis pullastra*.

CHAPTER 5 – Physiological responses to depuration and transport of native and exotic clams at different temperatures

Table 1. Summary of criteria used for scoring behavioral clam activity.

Table 2. Results of two-way ANOVA evaluating the effects of transport temperature (4 and 22 °C) and species (*Venerupis pullastra* and *Ruditapes philippinarum*) on survival, CI, K and A.E.C. values and glycogen content.

Table 3. Pearson correlation coefficients between survival, CI, ATP-related compounds (IMP, ATP, ADP, AMP, Hx and HxR), K and A.E.C. values and glycogen content in each treatment of *Ruditapes philippinarum*.

Table 4. Pearson correlation coefficients between survival, CI, ATP-related compounds (IMP, ATP, ADP, AMP, Hx and HxR), K and A.E.C. values and glycogen content in each treatment of *Venerupis pullastra*.

Table 5. Results of two-way ANOVA evaluating the effects of transport temperature (4 and 22 °C) and species (*Venerupis pullastra* and *Ruditapes philippinarum*) on ATP-related compounds (IMP, ATP, ADP, AMP, Hx and HxR).

CHAPTER 6 – **Effects of depuration on metal levels and health status of bivalve molluscs**

Table 1. Elemental concentration (mg/kg dw; n = 4) and detection limits (mg/kg, D.L.) of certified reference materials (average ± standard deviation) analysed by FAAS and EDXRF.

Table 2. Biometric data (length, width, height, total weight, edible weight and total meat yield), condition index (CI) and cumulative mortality rate (%) during the depuration time of the three bivalve species (RP, MG and SP).

Table 3. Elemental composition (mean ± standard deviation; wet weight) of RP, MG and SP during the depuration time: macro elements (S, Cl, K and Ca; mg/kg, ww) and trace elements (Fe, Zn, Br Cu, Se, Rb and Sr; mg/kg, ww).

Table 4. Effect of depuration time on change in concentration (% reduction/increase) of macro (S, Cl, K and Ca), trace (Fe, Zn, Br, Cu, Se, Rb and Sr) and toxic elements (Hg, Cd, Pb and As) of RP, MG and SP.

Table S1. Results of two-way ANOVA evaluating the effects of depuration time and species (RP, MG and SP) on glycogen content, macro (S, Cl, K and Ca), trace (Fe, Zn, Br, Cu, Se, Rb and Sr) and toxic elements (Hg, Cd, Pb and As) concentration.

Table S2. Results from Pearson correlation coefficients between macro, trace and toxic elements of RP, MG and SP.

CHAPTER 7 – **Ecophysiology of native and alien invasive clams in an ocean warming context**

Table 1. Results of two-way ANOVA evaluating the effects of temperature and species (*Ruditapes decussatus* and *Ruditapes philippinarum*) on routine metabolic rates (RMRs), heat shock response (HSP70/HSC70 levels), lipid peroxidation (MDA levels) and glutathione S-transferase (GST), catalase (CAT) and superoxide-dismutase (SOD) activities.

CHAPTER 8 – **Effect of warming on protein, glycogen and fatty acid content of native and invasive clams**

Table 1. Fatty acid quantitative composition (mg/g dw) of the native (*Ruditapes decussatus*) and invasive clams (*Ruditapes philippinarum*) at different temperatures.

CHAPTER 9 – General discussion and conclusions

Table 1. Main risks associated with bivalve molluscs' consumption and higher-risk population.

Table 2. Seasonal bacterial variation (*E. coli*, *Vibrio* spp., TVC and *Salmonella* spp.) in clams (*Venerupis pullastra* and *Ruditapes philippinarum*), water and sediment from Trafaria and Barreiro.

Table 3. Microbiological responses (TVC, *E. coli* and *Vibrio* spp. levels) of native (*Venerupis pullastra*, VP) and invasive clams (*Ruditapes philippinarum*, RP) to depuration and semidry simulated transport at two temperatures (4 and 22 °C).

Table 4. Significant reduction/increase (%) on concentration of essencial and toxic elements of three bivalves' species depurated (*Ruditapes philippinarum*, RP, *Mytilus galloprovincialis*, MG and *Scrobicularia plana*, SP).

Table 5. Physiological responses (mortality, condition index, breakdown products of ATP and glycogen content) of native (*Venerupis pullastra*, VP) and invasive clams (*Ruditapes philippinarum*, RP) to depuration and semidry simulated transport at two temperatures (4 and 22 °C).

Table 6. Physiological (CTMax, RMRs, Q_{10} values, HSP70/HSC70 levels, MDA and antioxidant enzyme activities) and biochemical responses (protein, glycogen and fatty acids contents) of native (*Ruditapes decussatus*, RD) and invasive clams (*Ruditapes philippinarum*, RP) to environmental warming.

ABSTRACT

Bivalves are extremely important in Portuguese economy and gastronomy, being clams the main species consumed. Nevertheless, bivalve consumption raises specific food safety concerns, particularly due to the accumulation of biological contaminants, including microbial and chemical, as they are filter-feeders inhabiting estuaries constantly subjected to contamination and climate variability. In this context, the main objectives of this dissertation were to investigate the bivalve consumption behaviour, to compare the microbiological, chemical and physiological responses of native and invasive clams to depuration and transport in semi-dry conditions at optimal and stressful temperatures, and to assess the impact of warming in bivalve metabolism, oxidative stress and nutritional quality. Bivalve consumer's presented risky behaviours that differed according to their demographic and socio-economic characteristics. Higher bacterial levels were observed in invasive species, regardless of season and environmental parameters (higher *Escherichia coli* and total viable counts in winter and *Vibrio* spp. in summer). Depuration was an efficient process to reduce the levels of *E. coli* and toxic elements (particularly Pb) in different bivalves to levels considered as acceptable for human consumption, but not to eliminate *Vibrio* spp. Transport at 4 °C was indeed the best solution to maintain good physiological conditions of clams and to avoid the *Vibrio* spp. growth. Despite invasive clam species demonstrated higher survival at lower temperatures compared to the native ones, they also revealed similar upper thermal tolerance limits. Distinct physiological requirements and mechanisms of defence against warming were observed: while the invasive closes the valves, the native uses glycogen and fatty acids as energy sources, and displayed higher metabolism, activity of antioxidant enzymes, heat shock proteins and lipid peroxidation. Overall, the main outputs of this dissertation allowed to correctly predict the risks associated with bivalve consumption to greatly improve the knowledge of stress physiology in native and invasive clams.

Keywords: native and invasive clams; microbial and chemical contamination; physiology; depuration; transport; warming.

RESUMO

Os bivalves são extremamente importantes na economia e gastronomia portuguesa, sendo as amêijoas as principais espécies consumidas. Contudo, o seu consumo levanta preocupações acrescidas de segurança alimentar devido à acumulação de contaminantes biológicos, incluindo os microbiológicos e químicos, dado que são organismos filtradores que habitam em estuários constantemente sujeitos a contaminação e variabilidade climática. Neste contexto, os objetivos desta dissertação foram investigar o comportamento dos consumidores de bivalves, comparar as respostas microbiológicas, químicas e fisiológicas de amêijoas nativas e invasoras à depuração e transporte em condições semi-secas a diversas temperaturas, e avaliar o impacto do aquecimento no metabolismo, stress oxidativo e qualidade nutricional. Os consumidores de bivalves apresentaram comportamentos de risco que diferem de acordo com as suas características demográficas e socioeconómicas. Foram observados níveis mais elevados de bactérias nas espécies invasoras, independentemente da época do ano e parâmetros ambientais (*Escherichia coli* e contagens totais de viáveis no inverno e *Vibrio* spp. no verão). A depuração revelou-se eficiente na redução dos níveis de *E. coli* e elementos tóxicos (particularmente Pb) em diferentes bivalves para níveis considerados aceitáveis para consumo humano, mas não é eficiente na eliminação do *Vibrio* spp. O transporte a 4 °C permitiu melhores condições fisiológicas das amêijoas e evitou o crescimento de *Vibrio* spp. Apesar das amêijoas invasoras demonstrarem maior sobrevivência a temperaturas baixas em relação às nativas, mostraram igualmente limites de tolerância térmica superiores similares. Distintos requisitos fisiológicos e mecanismos de defesa contra o aquecimento foram observados: enquanto a invasora fechou as valvas, a nativa usou glicogénio e ácidos gordos como fontes de energia, e revelou maiores taxas metabólicas, atividades de enzimas antioxidantes, proteínas de choque térmico e peroxidação lipídica. Os resultados desta dissertação permitem compreender os riscos associados ao consumo de bivalves e os mecanismos fisiológicos de tolerância ao stress em amêijoas nativas e invasoras.

Palavras-chave: amêijoas nativas e invasoras; contaminação microbiológica e química; fisiologia; depuração; transporte; aquecimento.

RESUMO ALARGADO

Os moluscos bivalves são muito apreciados pelos consumidores Europeus tanto do ponto de vista gastronómico como socioeconómico. Estes organismos apresentam um elevado valor nutricional contribuindo para uma dieta saudável. Contudo, sendo organismos filtradores, podem acumular bactérias patogénicas, vírus, contaminantes químicos tóxicos, entre outros, constituindo um potencial perigo de saúde pública. Assim, torna-se necessário desenvolver estratégias de monitorização e assegurar uma fiscalização rigorosa ao longo do seu circuito de comercialização, de modo a tornar estes produtos seguros para o consumidor. Por outro lado, sendo as alterações climáticas uma realidade, é expectável que num futuro próximo o aquecimento dos oceanos e zonas costeiras possa vir a afetar a sobrevivência e os processos fisiológicos dos bivalves. Neste contexto, este estudo teve como principais objetivos: a) conhecer o perfil dos consumidores de bivalves e os seus comportamentos de risco; b) analisar a influência da época do ano e dos parâmetros ambientais na ocorrência de bactérias patogénicas em espécies nativas e invasoras; c) avaliar e comparar as respostas microbiológicas, químicas e fisiológicas de espécies de bivalves em duas etapas críticas do seu circuito de comercialização, nomeadamente a depuração e o transporte em condições semi-secas a diversas temperaturas; e d) avaliar o impacto do stress térmico no metabolismo, stress oxidativo e qualidade nutricional de duas espécies de amêijoas.

Do inquérito elaborado a nível nacional, verificou-se que as amêijoas são as espécies mais consumidas, nomeadamente *Venerupis pullastra*, *Ruditapes decussatus* e *Ruditapes philippinarum*, as quais estão também entre as principais espécies capturadas no estuário do Tejo. Contudo, estes bivalves podem representar um perigo para a saúde pública devido a diversos comportamentos de risco dos consumidores: captura de especímenes em áreas poluídas ou compra direta aos pescadores, muitas vezes sem informação sobre o local de colheita; consumo de amêijoas cruas ou levemente cozinhadas ao vapor, e consumo de animais com conchas partidas e/ou fechadas após cozinhadas. As atitudes e preferências dos consumidores portugueses variaram consideravelmente de acordo com as suas características demográficas e socioeconómicas. Assim, os consumidores mais jovens (<25 anos), do sexo masculino, com nível de educação básica, com baixos rendimentos mensais do agregado familiar (≤800€) e que habitam junto ao litoral estão entre os consumidores com maiores comportamentos de risco em relação aos bivalves.

Da comparação realizada entre espécies nativas (*V. pullastra*) e invasoras (*R. philippinarum*) provenientes do estuário do Tejo, verificou-se que esta última apresentou

maior nível de contaminação microbiológica, nomeadamente *Escherichia coli*, *Vibrio* spp., *Salmonella* spp. e contagens totais de viáveis (CTV), refletindo a menor qualidade das águas e sedimento oriundo do interior do estuário do Tejo. Além disso, constatou-se que a ocorrência destas bactérias em ambas as amêijoas é influenciada pela época do ano e por parâmetros ambientais. Assim, os níveis mais elevados de *Vibrio* spp. foram encontrados no verão, principalmente na espécie invasora e nas amostras de sedimento, coincidindo com níveis baixos de precipitação e elevada salinidade e temperatura, enquanto os níveis mais elevados de *E. coli* e CTV foram observados durante o outono-inverno, particularmente nas amostras de água e sedimento, quando ocorreram maiores teores de oxigénio dissolvido, pH e precipitação, assim como temperatura e salinidade mais baixa. Relativamente à presença de *Salmonella* spp., que ocorreu em ambas as espécies de amêijoas e no sedimento do Barreiro, coincidiu geralmente com níveis elevados dos indicadores de contaminação fecal (*E. coli*).

Tendo em conta que ambas as espécies de amêijoas mostraram níveis de *E. coli* acima do limite legislado (230 unidades formadoras de colónias/100 g de peso húmido) para a área de produção de bivalves de categoria A, investigou-se se a depuração seria um processo eficiente na redução das bactérias. Como seria de esperar, os níveis de *E. coli* e CTV foram reduzidos em 24 e 48h de depuração, respetivamente, contrariamente aos níveis de *Vibrio* spp. que aumentaram. Além disso, a depuração demonstrou ser igualmente eficiente na redução de elementos tóxicos, particularmente o Pb na lambujinha *Scrobicularia plana* (39 e 60% de redução após 2 e 8 dias, respetivamente) para níveis abaixo dos limites máximos permitidos. Os níveis de elementos tóxicos variaram entre as três espécies de bivalves (*R. philippinarum*, *Mytilus galloprovincialis* and *S. plana*) provenientes do estuário do Tejo, provavelmente devido à variabilidade da poluição existente junto às zonas de produção de bivalves e às características biológicas destas espécies (as amêijoas geralmente vivem enterradas no sedimento, enquanto os mexilhões são organismos intertidais). Em termos fisiológicos, a depuração não afetou as espécies de bivalves estudadas, exceto a *S. plana,* que utilizou todas as reservas bioquímicas (glicogénio), com o consequente aumento das taxas de mortalidade, particularmente após 4 dias (25%).

O transporte de bivalves vivos em condições ótimas, isto é a temperaturas baixas (4 °C) é extremamente importante para manter a qualidade e a salubridade destes produtos, embora atualmente se constate que em muitos casos as amêijoas são sujeitas a grandes variações de temperatura ao longo da cadeia de comercialização. De facto, constatou-se que, em termos microbiológicos, a qualidade das amêijoas *V. pullastra* e *R. philippinarum* foi assegurada a 4 °C, pois os níveis de *E. coli* diminuíram e os níveis de CTV aumentaram

lentamente, enquanto os níveis de *Vibrio* spp. mantiveram-se constantes. Em contraste, o transporte a temperaturas mais elevadas (22 °C) induziu o crescimento de *Vibrio* spp. e o aumento dos níveis de CTV. Constatou-se igualmente ser importante assegurar uma depuração prévia das amêijoas de modo a garantir a sua segurança para os consumidores, uma vez que em condições ótimas, se obtiveram níveis de CTV abaixo do limite recomendado (até 120h e 96h de transporte para a *V. pullastra* e *R. philippinarum*, respetivamente), teores de *E. coli* dentro dos limites de segurança para o consumo humano e níveis de *Vibrio* spp. constantes. Do ponto de vista fisiológico, verificou-se que ambas as espécies apresentaram melhores condições quando mantidas a 4 °C, i.e. maior sobrevivência (5 e 14 dias para a espécie nativa e invasora, respetivamente), redução lenta do índice de condição (IC), catabolismo lento da adenosina trifosfato (ATP) com consequente acumulação de hipoxantina e inosina, percentagem baixa do valor-K e da carga energética adenílica (CEA), e diminuição lenta do teor de glicogénio. Concluiu-se ainda que todos estes parâmetros, à exceção da CEA, são indicadores de stress úteis para avaliar o estado fisiológico destas espécies de amêijoa. A espécie nativa revelou geralmente melhores condições fisiológicas (IC e glicogénio mais elevados, mas valor-K e CEA mais baixos) do que a invasora (maior sobrevivência, especialmente a 4 °C).

A avaliação dos limites de tolerância térmica superiores da espécie invasora *R. philippinarum* e nativa *R. decussatus* revelou valores idênticos para as duas espécies. No entanto, ambas revelaram diferentes requisitos fisiológicos e mecanismos de defesa contra o stress térmico. Assim, a espécie nativa apresentou melhor qualidade nutricional, mas desenvolveu respostas celulares energeticamente mais dispendiosas para suportar o aumento de temperatura, através da utilização de glicogénio e ácidos gordos como fontes de energia. Além disso, apresentou maiores taxas metabólicas, atividade de enzimas antioxidantes (glutathiona-S-transferase, GST; catalase, CAT; e superóxido dismutase, SOD), proteínas de choque térmico (HSP70) e peroxidação lipídica (concentração de malonaldeído). Por outro lado, a espécie invasora apenas utilizou os ácidos gordos como fonte de energia, e recorreu ao encerramento das valvas como mecanismo de defesa, contra o stress térmico, conferindo-lhe uma proteção adicional às condições adversas e assegurando maiores sobrevivências. Em relação ao efeito do stress térmico na qualidade nutricional destas espécies de amêijoas, constatou-se que o maior efeito negativo foi a perda de ácidos gordos *n*-3 polinsaturados, nomeadamente os ácidos eicosapentanóico (EPA) e docosahexanóico (DHA), conduzindo a uma menor qualidade de ambas as espécies para os consumidores.

Este estudo permite compreender os potenciais riscos microbiológicos e químicos associados ao consumo de bivalves, tendo em conta a sua origem e condições usadas ao longo do circuito de comercialização. Esta análise é particularmente relevante para que os consumidores, produtores e comerciantes estejam atentos, informados e implementem as melhores práticas para garantir produtos de elevada qualidade. Além disso, as respostas fisiológicas das amêijoas durante a depuração, transporte e stress térmico permitem compreender as suas capacidades adaptativas.

LIST OF PAPERS

This thesis includes seven papers that are listed below, each corresponding to a chapter (2 to 8). Five of the papers are already published in peer-reviewed international journals and another two papers are *in press*.

CHAPTER 2: Portuguese consumers' attitudes and perceptions of bivalve molluscs
Patrícia Anacleto, Sara Barrento, Maria Leonor Nunes, Rui Rosa, António Marques
Published in *Food Control* (2014) 41: 168–177
DOI: 10.1016/j.foodcont.2014.01.017

CHAPTER 3: Microbiological composition of native and exotic clams from Tagus estuary: Effect of season and environmental parameters
Patrícia Anacleto, Sónia Pedro, Maria Leonor Nunes, Rui Rosa, António Marques
Published in *Marine Pollution Bulletin* (2013) 74(1): 116–124
DOI: 10.1016/j.marpolbul.2013.07.019

CHAPTER 4: Microbiological responses to depuration and transport of native and exotic clams at optimal and stressful temperatures
Patrícia Anacleto, Ana Luísa Maulvault, Milena Chaguri, Sónia Pedro, Maria Leonor Nunes, Rui Rosa, António Marques
Published in *Food Microbiology* (2013) 36(2): 365–373
DOI: 10.1016/j.fm.2013.07.002

CHAPTER 5: Physiological responses to depuration and transport of native and exotic clams at different temperatures
Patrícia Anacleto, Ana Luísa Maulvault, Sara Barrento, Rogério Mendes, Maria Leonor Nunes, Rui Rosa, António Marques
Published in *Aquaculture* (2013) 408–409: 136–146
DOI: 10.1016/j.aquaculture.2013.05.035

CHAPTER 6: Effects of depuration on metal levels and health status of bivalve molluscs
Patrícia Anacleto, Ana Luísa Maulvault, Maria Leonor Nunes, Maria Luísa Carvalho, Rui Rosa, António Marques
In press in *Food Control*

CHAPTER 7: Ecophysiology of native and alien invasive clams in an ocean warming context

Patrícia Anacleto, Ana Luísa Maulvault, Vanessa M. Lopes, Tiago Repolho, Mário Diniz, Maria Leonor Nunes, António Marques, Rui Rosa

Published in *Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology* (2014) 175: 28–37

DOI: 10.1016/j.cbpa.2014.05.003

CHAPTER 8: Effect of warming on protein, glycogen and fatty acid content of native and invasive clams

Patrícia Anacleto, Ana Luísa Maulvault, Narcisa Bandarra, Tiago Repolho, Maria Leonor Nunes, Rui Rosa, António Marques

In press in *Food Research International*

DOI: 10.1016/j.foodres.2014.07.023

CHAPTER 1

1. General introduction

1.1 Economic importance of bivalve molluscs

1.1.1 Worldwide

1.1.2 Portugal

1.2 Main benefits associated with bivalve consumption

1.3 Main risks associated with bivalve consumption

1.3.1 Bacteria

1.3.1.1 *Escherichia coli*

1.3.1.2 *Salmonella* spp.

1.3.1.3 *Vibrio* spp.

1.3.2 Viruses

1.3.3 Parasites

1.3.4 Chemicals contaminants

1.3.5 Toxins from harmful algal blooms (HAB)

1.3.6 Classification of bivalve molluscs harvesting areas

1.4 Trade chain of live bivalves in Portugal

1.4.1 Depuration

1.4.2 Transport

1.5 Stress physiology in bivalve molluscs

1.5.1 Thermal tolerance limits and metabolism

1.5.2 Differences between alien invasive and native species

1.5.3 Oxidative stress biomarkers

1.5.4 Heat shock response

1.6 General aims and thesis outline

1.7 References

1. GENERAL INTRODUCTION

1.1 Economic importance of bivalve molluscs

1.1.1 Worldwide

World bivalve mollusc production (from fisheries and aquaculture) has consistently increased in the last sixty years, growing from nearly 1 million tonnes in 1950 to about 14.5 million tonnes in 2011, contributing 9.4% of the total amount in 2011 (FAO, 2014). During this period, the average annual growth in bivalve production was approximately 4.2%, though considerably higher in early 1990s (Fig. 1a). Crustaceans followed the same trend of bivalves, ranging from 0.7 to 12 million tonnes in this period (average annual growth rate 4.5%), whereas cephalopods grew at a slower annual rate (average 2.2%), varying between 0.6 and 3.8 million tonnes (Fig. 1a). In 2011, bivalve molluscs from fisheries represented almost 10% of the total world fishery production, whereas farmed bivalves represented 21% in volume and 11% in value of the total world aquaculture production. Such differences are mainly attributed to two factors: the rapid growth in the aquaculture sector and a sharp increase in Chinese bivalve production (Pawiro, 2010).

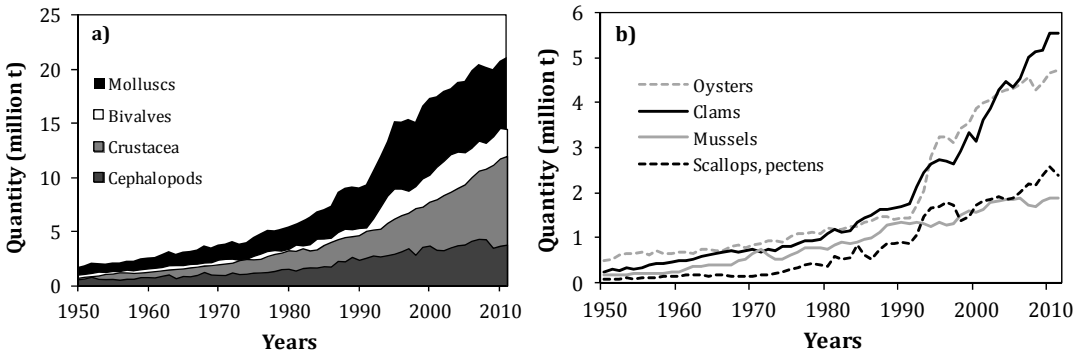


Figure 1. World production (fisheries and aquaculture) between 1950 and 2011 in tonnes (t) of the most important groups of seafood (a) and the most representative bivalve groups (b) (data source: FAO, 2014).

Global bivalve production from aquaculture has consistently increased over the years, mainly between 1990 and 2011 ranging from 3.3 to 12.8 million tonnes, respectively, with an average increase of 6.0% annually during this period. Aquaculture contribution to the overall bivalve production increased from 27.9% in 1950 to 87.8% in 2011, whereas bivalves from fisheries exhibited a downward trend during this period, declining from 72.1% to 12.2%.

Presently, the bulk of global bivalves production consists mainly of clams (38.1%, including cockles and ark shells) and oysters (32.5%) followed by scallops, pectens

(16.4%) and mussels (13.0%). However, until early 1990s the production of these species has steadily increased (Fig. 1b). It is noteworthy that the increased production of clams since the 1990s is largely due to the exponential growth of Japanese carpet shell, *Ruditapes philippinarum.* This is one of the most relevant species farmed worldwide, accounting with 3.7 million tonnes in 2011. Overfishing and irregular yields of the native (European) grooved carpet shell, *Ruditapes decussatus*, led to the introduction of *R. philippinarum* into European waters. Consequently, *R. philippinarum* populations are currently the major contributor to clam landings in Europe (FAO, 2014). Other bivalve species with significant world production are: constricted tagelus *Sinonovacula constricta* (745 thousand tonnes), Pacific cupped oyster *Crassostrea gigas* (673 thousand tonnes), blood cockle *Anadara granosa* (438 thousand tonnes), yesso scallop, *Patinopecten yessoensis* (426 thousand tonnes), Chilean mussel *Mytilus chilensis* (289 thousand tonnes), American sea scallop *Placopecten magellanicus* (281 thousand tonnes), blue mussel *Mytilus edulis* (235 thousand tonnes) and American cupped oyster *Crassostrea virginica* (192 thousand tonnes). The bulk of these species contribute with 23% of the world total bivalve production in 2011 (Fig. 2).

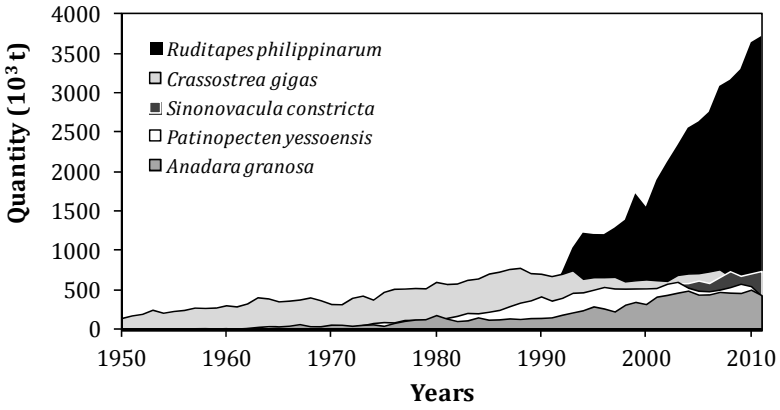


Figure 2. World production (fisheries and aquaculture) between 1950 and 2011 in tonnes (t) of main produced bivalves' species (data source: FAO, 2014).

China is by far the leading producer of bivalve molluscs with 10.5 million tonnes in 2011 and representing 71.9% of the global production and 81.9% of the global bivalve aquaculture production in that year, which is substantially due to the high production of clams (including cockles and ark shells) and oysters (8.4 million tonnes; Fig. 3). United States of America (USA) and Japan were the second and third largest producers, far behind China with a production of 698 and 656 thousand tonnes, respectively. Other main bivalve producing countries are Republic of Korea, Chile, Spain, France, Peru, Thailand, Canada, Italy and Mexico (Fig. 3).

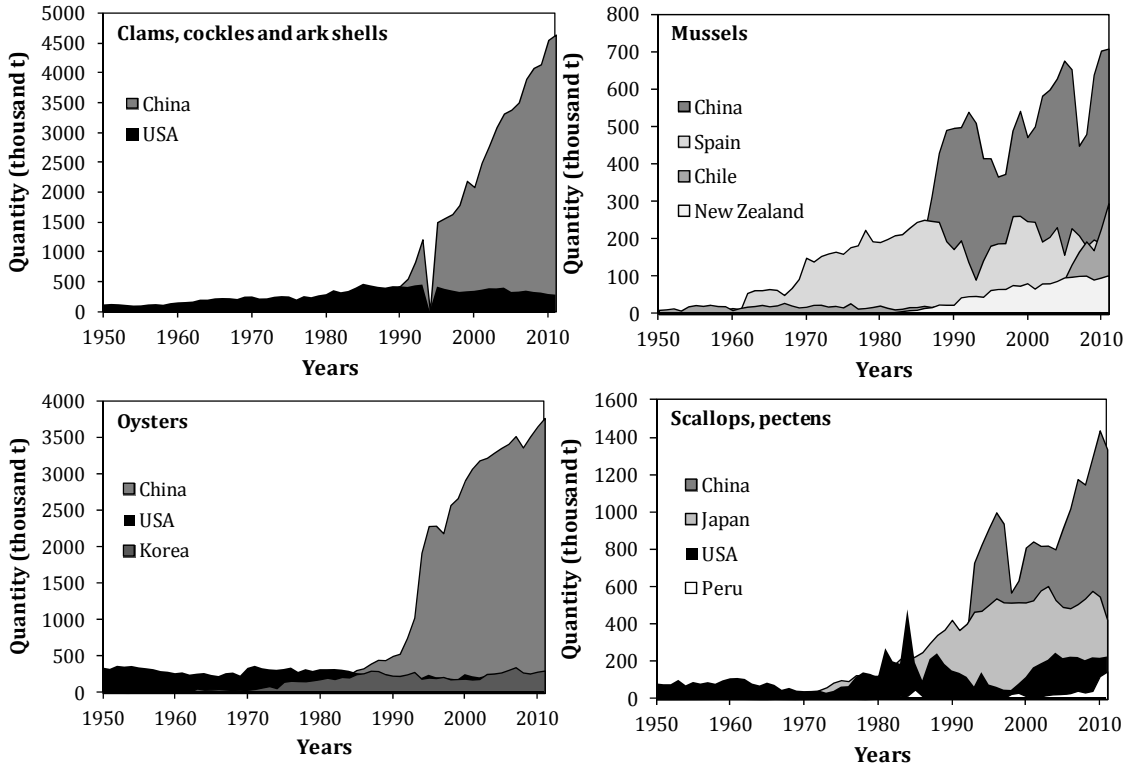


Figure 3. Major bivalve producing countries (fisheries and aquaculture) of different bivalve mollusc groups, data in tonnes (t) from 1950 to 2011 (data source: FAO, 2014).

1.1.2 Portugal

Portugal is the eighth biggest per capita seafood consumer worldwide and the highest in the European Union (EU), with an annual average of 61.1 kg, well above the average world per capita consumption (18.4 kg; 2009; FAO 2014). Among seafood, bivalve molluscs are greatly appreciated by consumers (2.5 kg/capita in 2009; INE/DGPA, 2010) from a social, gastronomic and economic point of view. Generally, bivalve species are preferably consumed live/raw (e.g. oysters) as a guarantee of freshness or lightly cooked (e.g. clams and mussels). Nowadays, bivalves' role in Portuguese gastronomy is increasing due to tourism, particularly in coastal regions (e.g. Ericeira, Sesimbra, Sines and Figueira da Foz), and to the popular shellfish festivals (e.g. Olhão, Faro, Setúbal, Ílhavo and Esposende). The Algarve tourism industry emphasizes gastronomic tourism as an important complement for the sustainability of the bivalve activity (WTTC, 2003).

The national production of bivalves has shown important progress in the last years, particularly clams and cockles (Fig. 4). The exception occurred with *C. gigas* that revealed a deep decrease in production in the last years after 10 years-period without any production (1976-1986). The main cause of this decline in the most productive areas of oysters was the industrial pollution, mainly attributed to the introduction and uses of

tributyltin (TBT) as an antifouling paint additive on ships to avoid the attachment of marine organisms in marine infrastructures (Rato, 2009).

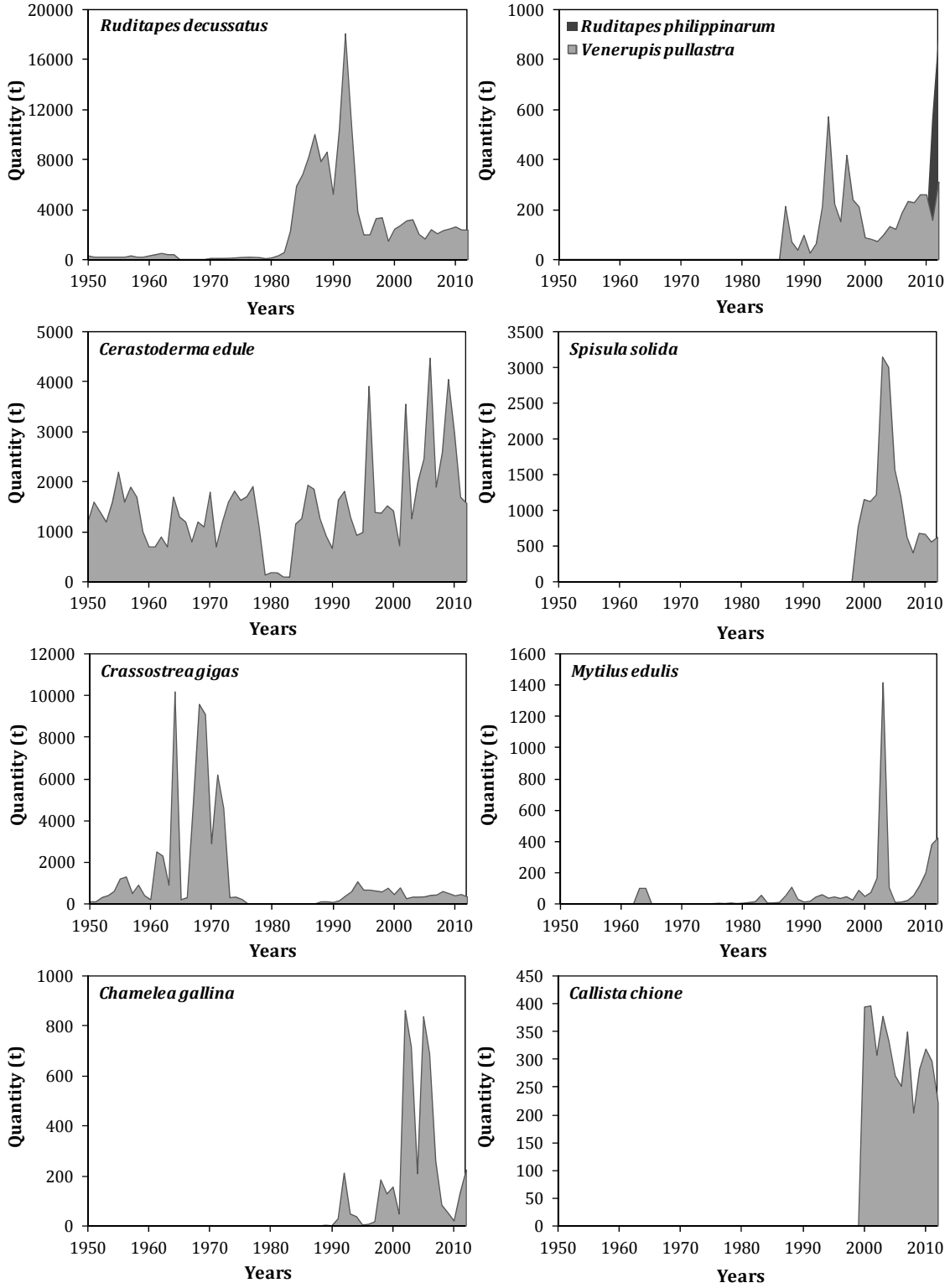


Figure 4. National production (fisheries and aquaculture) in tonnes (t) of the most representative bivalves from the Portuguese coast between 1950 and 2012 (data source: EUROSTAT, 2014; FAO, 2014).

Other factor was the occurrence of several epizootics, namely the "oyster gill disease" that is caused by an iridovirus (Comps *et al.,* 1976). This disease reduced the filtering capacity and killed young and stressed animals. Associated to this problem was the phenomenon of abnormal shell growth (Key, 1981). Additionally, the "foot disease", protozoan diseases, ineffective or absent management strategy to protect natural beds, overharvesting and non-existence of hatcheries, may also have been responsible for the oysters mortality in the latest years (Ruano, 1997).

During the last years, *R. decussatus* and the common edible cockle *Cerastoderma edule* greatly contributed to national bivalve production (representing 29.7% and 19.7%, respectively of the total bivalve production in 2012), followed by *R. philippinarum* (11.1%), solid surf clam *Spisula solida* (7.8%), *M. edulis* (5.3%), *C. gigas* (4.2%), pullet carpet shell clam *Venerupis pullastra* (3.9%), striped venus *Chamelea gallina* (2.8%) and smooth callista *Callista chione* (2.8%; Fig. 4).

Since early 1990s, the national bivalve demand and consumption have increased to a level where production is not sufficient, and therefore imports usually exceed exports with an inevitable negative trade balance, i.e. a deficit (Fig. 5; EUROSTAT, 2014). Total bivalve trade in Portugal has expanded continuously during the past three decades to reach € 24.9 million and € 19.2 million in imports and exports, respectively, in 2013, representing an increase of 3517% and 793%, respectively, during that period. By far the most important bivalve products in terms of import volume are traded preserved or processed (e.g. 89.3% in 2013; i.e. traded as frozen, dried, salted, in brine, smoked or cooked), but in terms of export volume bivalves are equally traded live, fresh or chilled (e.g. 48.2% in 2013), as well as preserved or processed (e.g. 51.8% in 2013).

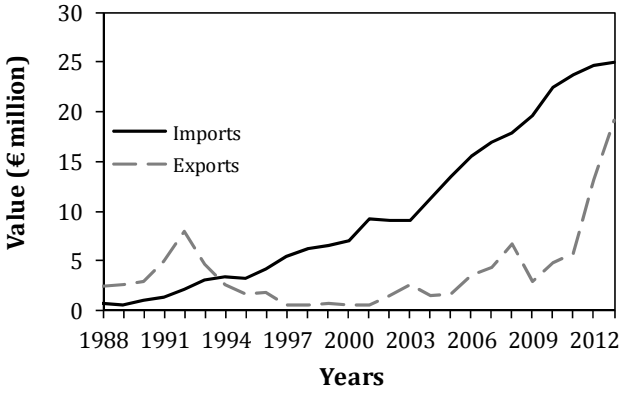


Figure 5. Imports and exports of bivalves by Portugal, data in value (€ million) from 1988 to 2013 (data source: EUROSTAT, 2014).

In terms of quantity and value, clams, cockles and ark shells are the most important bivalves imported, with an average growth rate per year of 13.5%. In 2013, clams accounted 74.6% and 61.5% of the total bivalve quantity and value, respectively, followed by mussels (22.2 and 30.3%), scallops/pectens (2.3 and 7.1%) and oysters (0.9 and 1.2%; Fig. 6). Clams are mainly supplied by Vietnam, Spain and Netherlands. Interestingly, preserved or processed clams are responsible by 72.9% of the total volume of national bivalve imports in 2013, which is particularly due to clams from Vietnam, representing 82.2% of all clams imported by Portugal (Fig. 6). On the other hand, the main import countries of mussels to Portugal are Spain, New Zealand and Netherlands, whereas the major importers of oysters to Portugal are France and Spain, and the most relevant importers of scallops and pectens to Portugal are Spain, Netherlands, France, Peru and Vietnam (Fig. 6).

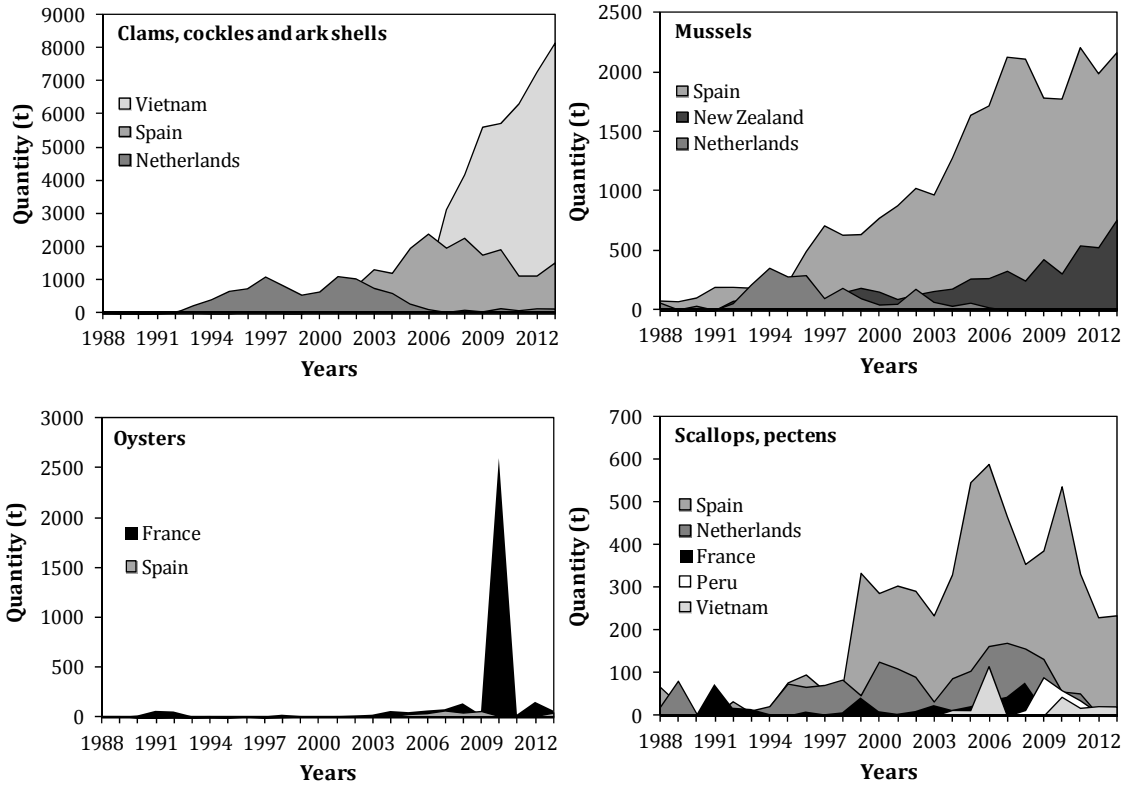


Figure 6. Main importers of bivalves from Portugal, data in tonnes (t) from 1988 to 2013 (data source: EUROSTAT, 2014). Note: Data includes bivalves with or without shell, live, fresh or chilled, frozen, dried, salted or in brine and also smoked and cooked in shell. The detailed classification codes used by Eurostat are presented in Annex.

Regarding exports, clams are the most important species in terms of quantity and value, accounting 73.0% and 59.7% in 2013, respectively, followed by mussels (17.3% and

29.2%, respectively; Fig. 7). The main target markets for exportation of bivalves harvested or produced in Portugal are western European countries, especially Spain and France. Until the late 1990s the main exported bivalves were scallops and pectens, whereas higher exports of clams, cockles and ark shells were registered since 2003, attaining a total of 5.5 thousand tonnes exported to other countries in 2013 and an income of € 11.5 millions. Live, fresh or chilled clams are the main exported bivalves, particularly to Spain (41.5%). Despite the increasing exportation registered during the last twenty years, the trade balance of bivalves is still negative, with a loss of € 5.7 million in 2013 (Fig. 5).

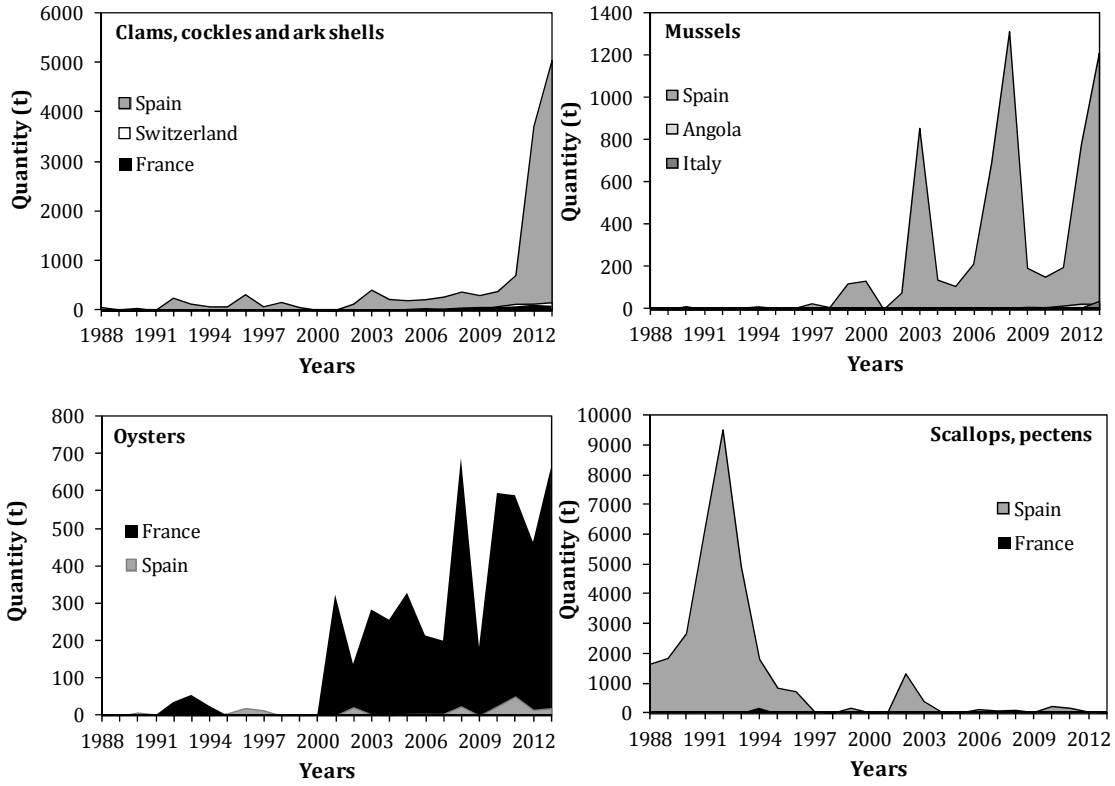


Figure 7. Main exporters of bivalves to Portugal, data in tonnes (t) from 1988 to 2013 (data source: EUROSTAT, 2014). Note: Data includes bivalves with or without shell, live, fresh or chilled, frozen, dried, salted or in brine and also smoked and cooked in shell. The detailed classification codes used by Eurostat are presented in Annex.

Overall, the trade of bivalves is mainly between Portugal and EU countries and only limited amounts of specific species are imported from third countries like Vietnam and New Zealand. Importing countries enforce strict regulations on live, fresh and frozen bivalves which many exporting non-EU countries are unable to meet (Pawiro, 2010). Under the EU import regulations on bivalves, currently only 13 third countries are authorized to export their bivalves to the EU markets (Pawiro, 2010). This contrasts with

other general seafood products, where approximately 100 third countries and territories have been approved to export their products to the EU (Pawiro, 2010).

1.2 Main benefits associated with bivalve consumption

Bivalve molluscs are nutritionally very important for human consumption, being considered as delicacies and healthy food items and, thus, playing a central role in the Portuguese gastronomy (Dong, 2001; Anacleto *et al.*, 2014). Their nutritional characteristics vary among species and even between individuals of same species, since they are generally influenced by age, sex, maturation stage, origin, season, seawater physical-chemical properties (e.g. temperature and pH) and feed composition and availability (Okumus and Stirling, 1998; Orban *et al.*, 2002).

Bivalve edible tissues are mainly composed by moderate protein (9-17%) and high carbohydrates contents (2.6-7.0%), but low lipid (0.8-2.3%), cholesterol (28-85 mg/100 g) and energetic values (243-440 kJ/100 g) (Table 1; Bandarra *et al.*, 2004; Silva and Batista, 2008). Carbohydrates content in bivalves are mainly stored as glycogen, exhibiting a clear seasonal variability related to species' reproductive cycle, reaching its lower peak immediately after spawning (Barber and Blake, 1981; Matias *et al.*, 2013). Despite considerable variations between bivalve species, fatty acids profile is particularly interesting, as the *n*-3 long-chain polyunsaturated fatty acids (PUFA) are generally predominant, such as eicosapentaenoic (EPA; 20:5*n*-3) and docosahexaenoic (DHA; 22:6*n*-3) acids (Table 1; Bandarra *et al.*, 2004; Silva and Batista, 2008), which are essential nutrients for marine organisms and humans (Sargent *et al.*, 1999). These fatty acids play a major role in the prevention and recovery from cardiovascular diseases, asthma, diabetes, arthritis, cancer and foetal malformations (Simopoulos, 1991). Other fatty acids are also typically found at high concentrations in bivalves, such as palmitic acid (PA; 16:0), oleic acid (OA; 18:1*n*-9) and arachidonic acid (AA; 20:4*n*-6). The quality indices generally used to estimate the probability of developing coronary heart diseases are *n*-3PUFA/*n*-6PUFA ratio, PUFA/SFA ratio, atherogenic (AI) and thrombogenic (TI) indices, which consider the beneficial aspects of unsaturated lipids (MUFAs and PUFAs), opposed to the adverse effects of saturated ones (SFA) (Ulbricht and Southgate, 1991; HMSO, 1994). Hence, bivalve species evidence balanced ratios of PUFA/SFA and *n*-3PUFA/*n*-6PUFA, as the minimum recommend values are exceeded for most species (0.45 and 0.25, respectively; HMSO, 1994). Recommendations regarding AI and TI have not yet been proposed, though the values obtained with bivalves are similar to those found in other seafood products. The presence of free amino acids, such as threonine, glycine and alanine, confers the

characteristically sweet taste of bivalves, whereas glutamic and aspartic acids are responsible for the acidic taste, and aspartate and glutamate are associated to the *umami* taste, also described as meat flavor (Bandarra *et al.*, 2004). Other components responsible for the sapidity of shellfish are also found, such as non-protein nitrogen compounds (e.g. free amino acids, nucleotides and volatile compounds; Bandarra *et al.*, 2004).

Table 1. Biochemical composition of the most consumed bivalve molluscs in Portugal.

	Oyster (*Crassostrea spp.*)	**Mussel** (*Mytilus spp.*)	**Clam** (*Ruditapes spp.*)	**Scallop** (*Pecten spp.*)
Nutritional data (/100g)				
Energetic value (kcal/kJ)	81/339	86/360	58/243	105/440
Lipids (g)	2.3	2.2	0.9	0.8
Proteins (g)	9.4	11.9	11.7	17.1
Carbohydrates(g)	4.9	7.0	2.6	6.0
Cholesterol (mg)	50	28	44	85
Macro and trace elements (mg/100g)				
Ca	270	37	51	31
Mg	22	34	103	48
Fe	17	2	9	3
Na	106	286	244	301
K	234	171	78	250
Mn	0.60	0.34	0.65	0.00
Cu	2.1	0.14	0.18	0.20
Zn	16.8	1.3	2.1	0.80
Se	0.07	0.04	0.02	0.04
Toxic elements (mg/kg)				
Hg	0.04	0.02	0.02	-
Cd	0.30	0.13	0.02	-
Pb	0.10	0.20	0.10	-
As	1.8	1.7	4.1	3.1
Essential amino acids (g/100g)				
Histidine	0.2	0.2	0.3	0.3
Isoleucine	0.4	0.5	0.6	0.7
Leucine	0.7	0.8	0.9	1.2
Lysine	0.7	0.9	1.0	1.3
Methionine+Cysteine	0.3	0.5	0.1	0.6
Phenylalanine+Tyrosine	0.6	0.8	0.9	1.1
Threonine	0.4	0.5	0.6	0.7
Valine	0.4	0.5	0.6	0.7
Fatty acids (%)				
14:0	10.5	4.4	0.3	4.8
16:0	15.7	17.3	16.0	28.5
18:0	3.6	2.9	6.9	8.0
ΣSFA	35.1	28.7	27.5	43.3
Σ16:1	6.7	9.6	3.3	4.4
Σ18:1	8.9	4.2	5.5	8.6
Σ20:1	5.2	5.6	4.1	3.3
ΣMUFA	23.6	22.5	20.2	18.6
18:2*n*-6	2.2	1.3	0.7	1.7
18:3*n*-3	2.8	1.2	0.4	1.2
20:4*n*-6	6.5	1.8	3.0	2.4
20:5*n*-3	13.6	15.2	5.3	7.7
22:6*n*-3	5.1	11.6	17.5	14.9
ΣPUFA	41.5	45.7	41.8	38.1
ΣPUFA/ΣSFA	1.18	1.59	1.60	0.88
n-3PUFA/*n*-6PUFA	2.83	8.62	3.97	5.27
AI	0.91	0.52	0.29	0.94
TI	0.28	0.17	0.21	0.42

- Lack of data; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; AI: Atherogenic index; TI: Thrombogenic index. Adapted from Bandarra *et al.*, 2004; Lourenço *et al.*, 2006; Silva and Batista, 2008; Telahigue *et al.*, 2010; DTU, 2014.

Bivalves are also important sources of macro and trace elements, as they contain almost all essential elements in their edible parts. However, the content varies according to several biological and environmental parameters, including species (0.8-3.0%) and origin (Silva and Batista, 2008). Generally, the most abundant elements are potassium (K), phosphorus (P; data not shown), sodium (Na), calcium (Ca) and magnesium (Mg). Some bivalve species, such as oysters, can also provide good intakes of zinc (Zn) and iron (Fe). Within toxic elements, arsenic (As) is the most relevant element, although the levels in bivalves are generally lower than those observed in other seafood species, and mostly in the non-toxic organic form (Lai *et al.*, 1999). Concerning vitamins, despite some variations are found within species, relevant contents of vitamins A (high in mussels), B_{12} (high in oysters), B_6, B_1 and niacin can be found in bivalves (data not shown; Bandarra *et al.*, 2004; Silva and Batista, 2008).

1.3 Main risks associated with bivalve consumption

Bivalve molluscs are filter-feeder organisms that selectively filter small particles of phytoplankton, zooplankton and inorganic matter, and can accumulate a diversity of other contaminants from the surrounding seawater (Burkhardt and Calci, 2000; Lees, 2000; Huss *et al.*, 2003). The main hazards associated with the consumption of bivalve molluscs arise from the contamination of water in which they grow, especially when they are intended to be eaten raw or lightly cooked as a whole (including hepatopancreas, their gastrointestinal tract) (Lees *et al.*, 2010). These circumstances make them an important vector of foodborne diseases, thus representing a significant human health risk (Lees *et al.*, 2010). These hazards include infections due to pathogenic bacteria, viruses, and parasites, as well as intoxications due to chemical (e.g. heavy metals and organic compounds) and toxins from harmful algal blooms (HAB; Table 2; Lee *et al.*, 2008a). The population with higher risk include individuals with immunosuppressive disorders (e.g. cancer patients, AIDS), achlorhydria and epilepsy, patients with diabetes mellitus, liver and chronic kidney diseases and steroid dependent patients (e.g. for treatment of asthma). Pregnancy, age and alcohol abuse are also factors that may enhance the risks of occurrence of shellfish diseases (Ripabelli *et al.*, 1999; Butt *et al.*, 2004). Compared to fish, bivalve molluscs are responsible for almost the double of patients, despite being responsible for a much lower number of outbreaks (Olsen *et al.*, 2000; Huss *et al.*, 2003). Between 2000 and 2010, most notifications from the European Union Rapid Alert System for Food and Feed (RASFF) were recorded for bivalve molluscs contaminated with *Escherichia coli* (36.1%), toxins from harmful algal blooms (HAB; 24.9 %), *Salmonella* spp. (10.5%), norovirus (8.1%),

heavy metals (6.7%) and *Vibrio parahaemolyticus* (0.4%; SARF, 2010). The next section briefly describes the main hazards that occur in bivalve molluscs.

Table 2. Hazards associated with bivalve molluscs' consumption (Lee *et al.*, 2008a).

Class of hazard	Contaminant
Infections	
Bacteria	*Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio cholerae*, *Campylobacter* spp., *Listeria monocytogenes*
Viruses	Norovirus, hepatitis A virus
Parasites	*Cryptosporidium* spp., *Giardia lamblia*, *Toxoplasma gondii*
Intoxications	
Chemical	Heavy metals: including mercury (Hg), cadmium (Cd), lead (Pb) Organics: dioxins, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pesticides
Toxins from harmful algal blooms (HAB)	Paralytic shellfish poisoning (PSP), diarrhoeic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP)

1.3.1 Bacteria

The number and type of pathogenic bacteria present in marine or estuarine waters depend on seasonal, climatic and anthropogenic factors (Vernocchi *et al.*, 2007). The bacteria found in bivalves can be divided in three groups (Reilly and Käferstein, 1997): (i) indigenous bacteria that naturally occur in marine or estuarine environments (e.g., *Vibrio* spp., *Listeria monocytogenes*, *Clostridium botulinum*, *Aeromonas hydrophila* and *Pleisomonas* spp.); (ii) non-indigenous/enteric bacteria that occur due to faecal contamination (e.g. *Salmonella* spp., *E. coli*, *Shigella* spp., *Campylobacter* spp. and *Yersinia enterocolitica*); and (iii) bacteria from cross-contamination during food preparation and processing (e.g., *Bacillus cereus*, *Stapylococcus aureus* and *Clostridium perfringens*). The sources of faecal contamination in bivalve harvesting areas can be punctual or diffuse. The most significant sources are sewage discharges, including sewage outfall, combined sewer overflows, recreational ports and rainwater discharges. The runoffs from agricultural activities combined with urban sources also play an important role in such contamination (Touron *et al.*, 2007; Oliveira *et al.*, 2011).

The retention of bacteria by bivalves depends on several factors, such as its form and dimension that influence bacteria adsorption and capture (Silva and Batista, 2008). The feeding physiology of bivalves also determines the accumulation of pathogenic bacteria filtered from the overlying water (Burkhardt and Calci, 2000), which may partially explain seasonal and geographical differences in bacterial content in bivalve species (Hernroth *et al.*, 2002). Environmental parameters, such as temperature, salinity, dissolved oxygen and turbidity can limit the filtration activity, also conditioning the retention of bacteria by bivalves (Silva and Batista, 2008). Therefore, bacteria may accumulate in bivalve species

at higher concentrations than those in the environment where they inhabit (Silva and Batista, 2008).

The accumulation factor is a measure generally used to assess sanitary risk associated with bivalve consumption and corresponds to the ratio between the geometric mean indicator concentration of bacteria in bivalves and the geometric mean concentration of bacteria in the overlying water (Derolez *et al.*, 2013; Kershaw *et al.*, 2013). Significant inter-species variations in baseline levels of bacteria have been reported in literature for several species. For example, the range of *E. coli* accumulation factors was similarly higher in cockles (*Cerastoderma edule)* than in mussels (*Mytilus* spp.), Pacific oysters (*Crassostrea gigas*), hard clams (*Mercenaria mercenaria)* or striped venus (*Chamelea gallina*) (Table 3).

Table 3. Examples of *E. coli* accumulation factors in bivalve species.

Species	Bacteria	Accumulation factor	References
Mussels (*Mytilus* spp.)	*E. coli*	15.2	Kershaw *et al.* (2013)
Pacific oysters (*Crassostrea gigas*)		11.7	Kershaw *et al.* (2013)
Common cockle (*Cerastoderma edule)*		330	Kershaw *et al.* (2013)
Hard clams (*Mercenaria mercenaria)*		6.5 – 8.5	Cabelli and Heffernan (1970)
		2.0 (0.02 – 17.5)	Burkhardt *et al.* (1992)
		3.0	Timoney and Abston (1984)
Striped venus (*Chamelea gallina*)		1.6	Martinez-Manzanares *et al.* (1991)

Only the bacteria studied in this work (*E. coli*, *Salmonella* spp. and *Vibrio* spp.) are highlighted below.

1.3.1.1 *Escherichia coli*

Escherichia coli is a faecal coliform bacterium (the predominant member of this group) that is defined as belonging to the enteric bacteria group and is widely distributed in the intestines of healthy humans and mammals. *E. coli* bacterium presence in bivalves and overlying waters is used as an indicator of recent faecal contamination, and therefore, the possible presence for pathogenic bacteria (Rhodes and Kator, 1998). Nonetheless, its absence does not necessarily guarantee the quality of water (Dutka, 1973). It is also the only biotype of the family *Enterobacteriaceae* that is exclusively from faecal origin (Warnes and Keevil, 2004). Although most strains of *E. coli* are not regarded as pathogens, they can be opportunistic pathogens that cause infections in immunocompromised hosts (Feng *et al.*, 2002).

1.3.1.2 *Salmonella* spp.

Bivalve harvesting areas may accumulate *Salmonella* spp. in contaminated waters. Particularly raw oysters have been the cause of salmonellosis outbreaks in USA. The disease is characterized by enteric (or typhoid) fever along with gastroenteritis, abdominal cramps and diarrhea (Brands *et al.*, 2005). The most common salmonella serotype that cause infection and death is *Salmonella* enterica serovar *Enteritidis*, followed by S. serovar *Typhimurium* (Heinitz *et al.*, 2000). *Salmonella* spp. survives better in colder temperatures (Wilson and Moore, 1996) and this pathogen is expected to become more widespread in the environment and hence throughout the food chain (Warnes and Keevil, 2004). However, detection of *Salmonella* in the absence of indicators of faecal pollution (*E. coli*) has also been reported (Dionísio *et al.*, 2000; Efstratiou and Tsirtsis, 2009).

1.3.1.3 *Vibrio* spp.

Among the indigenous microbiota of marine and estuarine water environments, the family *Vibrionaceae*, particularly *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae*, are the causative agent of human disease through the consumption of bivalve molluscs (Ripabelli *et al.*, 1999; Butt *et al.*, 2004; Normanno *et al.*, 2006). These natural pathogens remain viable and cultivable in water, even in the absence of organic matter (Croci *et al.*, 2002; Pruzzo *et al.*, 2005). Several studies from European countries reported the contamination of bivalves with *Vibrio* spp. (e.g. Høi *et al.*, 1998; Arias *et al.*, 1999; Ripabelli *et al.*, 1999; Potasman *et al.*, 2002). Some species are primarily associated with gastrointestinal illnesses (*V. parahaemolyticus* and *V. cholerae*), while others can cause non-intestinal illness, such as primary septicemia and severe wound infections (*V. vulnificus*) (FAO/WHO, 2002). The presence of *Vibrio* species is influenced by several factors such as temperature, salinity and algal density and there is no correlation between their occurrence and faecal human pathogens or related indicators (Huss *et al.*, 2003).

Vibrio parahaemolyticus has been recognized as an important cause of foodborne illnesses in Asian countries, with a high level of incidence in Japan (Alam *et al.*, 2002); and it is also recognized as the first cause of human gastroenteritis associated to shellfish consumption in United States (McLaughlin *et al.*, 2005). However, it has also been reported in some European countries (Hervio-Heath *et al.*, 2002; Martinez-Urtaza *et al.*, 2005; Roque *et al.*, 2009), but the risk of infections is considered to be low (EC, 2001; Su and Liu, 2007). Regarding *V. vulnificus*, the infections are mainly associated with the Gulf Coast area of the USA and are not common in Europe, although this microorganism has been isolated from other European areas such as the Italian Adriatic coast (Barbieri *et al.,* 1999). *V. cholerae* is

a frequent inhabitant of the coastal waters of the Bay of Bengal, but some outbreaks and sporadic cases of infection have been reported in USA (Klontz *et al.*, 1987). In these areas, characteristic and frequently seasonal epidemic peaks occur, being followed by periods of relative quiescence (Tirado *et al.*, 2010). The strains associated with the cholera illness (enterotoxigenic *V. cholerae* O1) are the most serious and debilitating (Potasman *et al.*, 2002).

V. parahaemolyticus and *V. vulnificus* are especially isolated in the summer months because of its high prevalence during warm seasons in estuaries (Oliver and Kaper, 1997), since most species are mesophilic organisms, i.e. they grows better in moderate temperatures (typically between 20 and 45 °C). The level of impact in the environment and within seafood products has been shown to be temperature dependent with rapid growth occurring at temperatures >16 °C (SARF, 2010). Wagley and Rangdale (2007) highlight the risk that global warming may lead to an increase in incidence of seafood associated food poisoning from *V. parahaemolyticus* in particular. On the other hand, during colder months, the organism probably survives in sediments and is then released into the water with zooplankton when the temperature rises (EC, 2001). Also, the highest numbers of this microorganism are seen at 20-25 PSU of salinity, as this species is a halotolerant bacterium (FAO/WHO, 2001). For example, *V. parahaemolyticus* is also capable of proliferation in live oysters during storage (increase 50 fold at 26 °C in 10 hours and almost 800 fold after 24 hours). However, low temperature storage generally causes a reduction by almost 10-fold during 14 days at 3 °C (Gooch *et al.,* 2002). In this sense, heat treatment (90 °C for 90 sec or equivalent) has been suggested as a very efficient method to easily eliminate most *Vibrio* spp., being this treatment included in the current EU directive for bivalves harvested in polluted waters (designated as category C areas; EC, 2004a). Additionally, the rapid and efficient cooling (time and temperature control) after harvesting, and the subsequent maintenance at low temperatures (less than or equal to 10 °C) is one of the most important control parameters in the prevention of *V. parahaemolyticus* proliferation and *V. vulnificus* gastroenteritis (Huss *et al.*, 2003; Lee *et al.*, 2008a). Concerning *V. cholera,* the main factors driving endemicity are a complex interplay of environmental and biological factors (Lipp *et al.*, 2002). Abiotic (environmental) factors, including temperature, salinity, iron concentration and sunlight, influence *V. cholera* toxin production, as well as the type of phytoplankton and zooplankton populations. This complex set of interactions impacts the survival and proliferation of *V. cholerae* in the estuarine environment (Tirado *et al.*, 2010).

1.3.2 Viruses

Regarding viruses that are more often linked to bivalve contamination and are associated with human illnesses are the norovirus (primarily called Norwalk-like virus, NLV), hepatitis A virus (HAV), enterovirus and adenovirus (Lees, 2000; Le Guyader and Atmar, 2007). Moreover, NLV and HAV are considered to be two of the main causes of gastroenteritis in humans due to their ability to induce infections with very low doses (1-10/PFU, plaque forming units) and by surviving in seawater for extended periods (Le Guyader *et al.*, 2009; Pinto *et al.*, 2009). Most of these viruses are also resistant to heat, disinfectants and pH changes (Silva and Batista, 2008). The common source of human viruses is derived from anthropogenic contamination, such as human sewage effluent waters and faeces into bivalve harvesting waters (Potasman *et al.*, 2002; Oliveira *et al.*, 2011). Since viruses may be concentrated in several tissues of the animal, they are difficult to eliminate by depuration (Lee *et al.*, 2008a). Consumers are often advised to consume raw bivalves during winter months when bacterial densities are low. However, the highest levels of viruses in shellfish have been observed in winter months (Burkhardt and Calci, 2000). Specifically, increases in norovirus infection have been associated with cold, dry temperature, low population immunity and novel genetic variants in Europe (Lopman *et al.*, 2009).

1.3.3 Parasites

The protozoan parasites *Cryptosporidium* spp*., Giardia duodenalis* and *Toxoplasma gondii* have been isolated in coastal waters and in bivalve molluscs (Robertson, 2007). These parasites life cycles involve fragile zoite stages that develop in the digestive tracts of animal hosts, including humans in some cases, and sturdy oocyst or cyst stages that are excreted through faeces and may be discharged to estuaries or coastal waters through surface runoffs from agricultural, suburban and urban land surfaces or improperly treated sewage and can accumulate and persist for prolonged periods in bivalves (Sinski, 2003; Robertson, 2007). So far the actual public health threat caused by parasites via bivalve consumption is unknown, largely because there is minimal epidemiological evidence of the infection transmission (Robertson, 2007).

1.3.4 Chemicals contaminants

Chemical contaminants, such as toxic elements (e.g. Hg, Cd and Pb), dioxins, furans, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) and pesticides are a potential hazard in certain bivalve harvesting areas (Lee *et al.*, 2008a). Among these environmental contaminants, toxic elements are a main concern due to their harmful effects on organisms, ability to bioaccumulate in aquatic ecosystems and low metabolization (Censi *et al.*, 2006). The filtration nature of bivalves lead to accumulation of high amounts of toxic elements, often having concentrations higher than in the sediment where they are buried (Karouna-Renier *et al.*, 2007) and, thus, easily reaching toxic concentrations to themselves and to consumers (Figueira *et al.*, 2011). For this reason, the level of toxic elements in bivalve molluscs raise public health concerns, and the European Commission has set Maximum Permissible Limits (MPLs) for toxic elements in edible tissues of bivalve molluscs (see Table 5 in point 1.3.6; EC, 2006). Mercury (Hg) is categorized among the most toxic substances found in the environment and can be detected in several chemical forms (Reyes *et al.*, 2008). Organically derived sources of Hg (e.g., methylmercury - MeHg) are frequently detected in fish and bivalve molluscs, and their toxicity is higher than inorganic sources (Kuballa *et al.*, 2009). After ingestion, Hg can rapidly enter in the circulatory system of humans and animals (Moore, 2000).

Concerning dioxins and dioxin-like compounds, they include a group of pollutants designated persistent organic pollutants (POPs), that are semi-volatile, persistent in the environment for long periods, bioaccumulative and highly toxic for humans (Loutfy *et al.*, 2007). The groups included in POPs are polychlorinated dibenzo-p-dioxins (PCDD) or polychlorinated dibenzofurans (PCDF), polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) which are mainly produced by anthropogenic activities as well as by natural processes (Loutfy *et al.*, 2007). Various toxic effects on immune, nervous, endocrine and reproductive systems and potential carcinogenic effects have been reported for some POPs groups (e.g. WHO, 1999; EC, 2002).

Pesticides are used especially in agriculture and some are harmful to humans or to the environment because of the extremely high toxicity, such as DDT (Dichloro-Diphenyl-Trichloro-ethane). Bivalves in harvesting areas may be affected by the presence of pesticides, potentially increasing their susceptibility to a wide range of infectious diseases (Renault, 2011). The effects of these contaminants may result from direct toxic actions on tissues or cells or from alterations of the homeostatic mechanisms, including the immune system (Coles and Pipe, 1994).

1.3.5 Toxins from harmful algal blooms (HAB)

HAB, also known as marine biotoxins or shellfish toxins, are mainly produced by species of naturally occurring micro-algal cells (phytoplankton) that are consumed by bivalves (Huss *et al.*, 2003). These toxins, usually linked to microalgae blooms, are not harmful to the bivalves, but might represent a risk to consumers safety even when bivalves are well-cooked, since toxins are heat resistant (Oliveira *et al.*, 2011). The toxins affect the central nervous system, but can also cause diarrhoea, vomiting, memory loss, paralysis and death (Huss *et al.*, 2003). The most relevant toxins from a public health point of view are the paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP) and diarrhoeic shellfish poisoning (DSP) (Lee *et al.*, 2008a). These toxins may cause respiratory and digestive problems, memory loss, seizures, lesions and skin irritation, or even fatalities in fish, birds, and mammals (including humans) (Sellner *et al.*, 2003). Some of these toxins can be acutely lethal, being some of the most powerful natural substances known without any antidote available to any harmful algal blooms toxin (Glibert *et al.*, 2005). The risk of toxic algae blooms show seasonal variability and the uptake, retention and depuration of toxins by bivalves vary greatly from one species to another (Lee *et al.*, 2008a; Shumway and Rodrick, 2009). Therefore, monitoring these toxins in bivalve harvesting areas is extremely important to prevent human intoxications as they are not destroyed when bivalves are frozen or heated (Lee *et al.*, 2008a).

1.3.6 Classification of bivalve molluscs harvesting areas

An evaluation of the sources and types of faecal contamination (from human and animal activities) in the vicinity of harvesting areas, combined with microbiological monitoring based on the use of indicator organisms (*E. coli* in the EU), provides an assessment of the risk of contamination with bacterial pathogens and is the basis for public health controls (Anonymous, 2012). In the EU, the classification of harvesting and production areas of bivalve molluscs, in terms of food safety, is specified in several Regulations: rules for the organisation of official controls (EC No. 854/2004; EC, 2004b), specific hygiene rules for the industry (EC No. 853/2004; EC, 2004a) and microbiological criteria (EC No. 1441/2007; EC, 2007).

From the microbiological point of view, the legislation employs a classification based on the levels of bacterial indicators of sanitary quality (*E. coli*) in bivalve flesh and intra-valvular liquid, quantified through a most probable number (MPN) test (reference method is ISO/TS 16649-3; ISO, 2005) and *Salmonella* spp.. The current classification determines if

bivalves can be chained for direct consumption or must be previously treated (Lees *et al.*, 2000). Table 4 summarises the EU classification criteria for bivalve molluscs harvesting areas and associated post-processing treatment required to reduce the risk to an acceptable level (EC, 2004b).

Table 4. European criteria for the classification of bivalve mollusc harvesting areas (EC, 2004b).

Category	*E. coli* level (MPN/100 g)	Post-harvest treatment required
A*	≤ 230	Direct human consumption
B	< 4600**	Depuration, relaying in an approved category A area or heat processing (cooking) by an approved method
C	≤ 46000	Relaying for at least 2 months in an category A or B area or heat processing (cooking) by an approved method
D (Prohibited)	> 46000	Harvesting prohibited

* includes also absence of *Salmonella* spp. in 25 g of flesh.

** live bivalve molluscs from these areas must not exceed, in 90% of samples, 4600 MPN *E. coli* per 100g of flesh and intra-valvular liquid. In the remaining 10% of samples, live bivalve molluscs must not exceed 46000 MPN *E. coli* per 100 g of flesh and intra-valvular liquid.

Commercial depuration is a controlled self-purification usually made in tanks of clean seawater for a period of time (more details in point 1.4.1), while relaying consists of transferring contaminated harvested bivalves to cleaner areas free of microbiological contamination allowing self-purification in the natural environment for longer periods (Richards, 1988; Lees, 2000). Levels of toxins from harmful algal blooms (HAB) and toxic elements in bivalve molluscs are also subjected to legal limits in order to minimize the risk to public health (Table 5). Above those limits harvesting cannot be undertaken.

Table 5. Regulatory levels for toxins from harmful algal blooms (HAB) and chemical contaminants in live bivalve molluscs (EC, 2004a, 2006).

Contaminants	Maximum levels
Toxins from harmful algal blooms (HAB)	
Paralytic shellfish poison (PSP)	800 µg of saxitoxin per kg
Amnesic shellfish poison (ASP)	20 mg of domoic acid per kg
Okadaic acid, dinophysistoxins and pectenotoxins together	160 µg of okadaic acid equivalents per kg
Yessotoxins	1 mg of yessotoxin equivalent per kg
Azaspiracids	160 µg of azaspiracid equivalents per kg
Chemical compounds	
Lead (Pb)	1.5 mg/kg wet weight
Cadmium (Cd)	1.0 mg/kg wet weight
Mercury (Hg)	0.50 mg/kg wet weight
Benzo(a)pyrene	10.0 µg/kg wet weight
Sum of dioxins	4.0 pg/g wet weight
Sum of dioxins and dioxin-like PCBs	8.0 pg/g wet weight

1.4 Trade chain of live bivalves in Portugal

The trade chain of live bivalve molluscs is complex and includes several steps, namely: 1) harvest/capture in bivalve growing areas; 2) transport; 3) grading; 4) depuration center, relaying area or industrial unit, depending on the classification of bivalve harvesting area; 5) dispatch center; 6) transport (national or international for export); 7) wholesaler/retailer; and 8) consumer. Figure 8 shows the different steps of this trade according to bivalve harvesting areas.

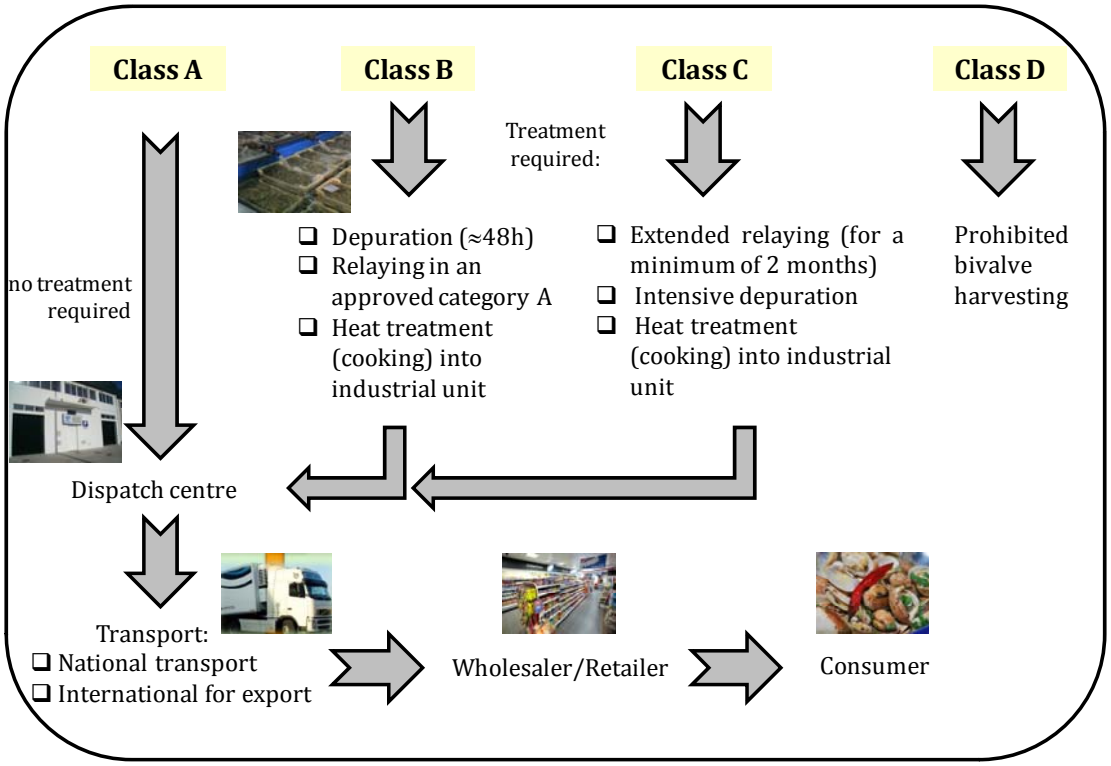


Figure 8. National trade chain of live bivalve molluscs from harvest to consumers (adapted from Bettencourt *et al.*, 2012).

From harvesting, different methods are commonly used, namely bottom trawls, dredges, snorkelling or digging (usually with a small pick) on shallow estuarine waters. The first two methods cause more physical disturbance and damage to animals, as well as a negative effect on the topography of the sediment surface. During this step, the handling procedures frequently used by fishermen, as well as the conditions onboard, are not optimal, since bivalves are frequently washed with freshwater, exposed to ambient temperature and direct sunlight during extended periods (generally between two to four hours). Bivalves are transported in boxes or net bags usually without temperature control during one to two hours to a grading facility to separate animals with poor quality (broken shells, gaping or with unpleasant smell) and dead animals. Afterwards, bivalves can be

transported once again (around one hour) to a depuration centre frequently located near coastal areas, being generally subjected to air exposure and wide temperature variations (i.e. not maintained in chilling conditions). At the holding facilities, bivalves are depurated (see conditions in point 1.4.1), packed and labelled for traceability, giving the confidence of a safe certified product to distributors and consumers (Jones *et al.*, 1991; Lees, 2000; Shumway and Rodrick, 2009). After packing and labelling, bivalves are placed in a cooling chamber during few hours to one day until dispatched to be processed by industries or transported to distributors (wholesalers or retailers; Figure 8; Bettencourt *et al.*, 2012). The transport between the dispatch centre and wholesalers or retailers is frequently made under recommended conditions, i.e. in trucks with chillers. Once at retailers, bivalves are placed on ice (stressful environment), strongly affecting animal condition. Ultimately, a final transport is performed by consumers, frequently under non-refrigerated conditions, after buying bivalves at retailer. Therefore, bivalve quality is strongly affected by traders and consumers' expectations, preferences and beliefs.

1.4.1 Depuration

Depuration (purification) is a process by which bivalve molluscs are maintained for a period of time that may range from several hours to days in a flow-through or recirculation system of chemically (through ozone, chlorination, iodophores, activated oxygen and iodophors) or physically (UV irradiation) disinfected water with sufficient oxygen and without any feed to reduce the content of potential pathogenic microorganisms (Lee *et al.*, 2008a). This process maximizes the natural filtering activity of bivalves, allowing the release of contaminants from their gills and intestinal tract and preventing the recontamination (Lee *et al.*, 2008a). Depuration has been applied to most bivalve species that are sold live, including clams, cockles, mussels, oysters and scallops and is practised throughout the world including in Europe, North America, Asia and Australia (Lees *et al.*, 2010). In Portugal, there are 28 legalized depuration and dispatch facilities, located in coastal areas throughout the country (DGPA, 2010). A number of requirements set by EU Regulations for the construction and general running of depuration centres cover elements such as tank construction and operation, the operation of batch systems, non-mixing of species during purification, hygiene of premises, approval of bivalve depuration and dispatch centres, laboratory testing, packaging, labelling, transportation, wet storage, and movement documentation/traceability requirements (Lees *et al.*, 2010).

Live bivalve molluscs that undergo depuration must be in good condition, because they are sensible animals that are susceptible to temperature extremes and physical shock. Therefore, it is vital to ensure that good harvesting and general handling practices are followed to avoid stressful conditions to the animals. During post-harvesting, the re-immersion of live bivalve molluscs should be avoided, except during depuration or controlled immersed storage, because when immersed, they will normally open and recommence filter-feeding and may accumulate any contaminants from the surrounding water (Lees *et al.*, 2010).

Depuration efficiency are influenced by several factors, such as the system design, initial water quality, oxygenation and flow rates, salinity, temperature, shellfish-to-water ratios, removal and settlement of faecal material, initial level of contamination, type and amount of pollutants, bivalve species and process duration (Jones *et al.*, 1991; Lee and Younger, 2002; Manfra and Accorneo, 2005; Barile *et al.*, 2009; Cozzi *et al.*, 2009). Among the several parameters, temperature and salinity are two of the most important to consider in the depuration process, according to the type of bivalve species, because influence time and efficiency of depuration (Table 6). Also, the maximum capacity and loading arrangements of standard design systems and maximum depth per tray for bivalve species commonly depurated are important to ensure good conditions. Artificially contaminated molluscs depurate more rapidly than environmentally contaminated ones (Richards, 1988; Jones *et al.*, 1991; Croci *et al.*, 2002) and different rates of elimination also occur when bivalves are contaminated with individual or mixtures of bacteria (Son and Fleet, 1980).

Table 6. Recommended temperature range, minimum salinity limit, permissible loading densities, and maximum depth per tray for depuration of different bivalve species (Lee *et al*., 2008a; Cefas, 2010).

Species	Temperature range (°C)	Minimum salinity (PSU)	Loading density	Maximum depth
Pacific oysters (*Crassotrea gigas*)	8 – 18	20.5	530 animals/m^2	Double layer
Native oysters (*Ostrea edulis*)	5 – 15	25.0	530 animals/m^2	Single layer overlapping
Mussels (*Mytilus edulis*)	5 – 15	19.0	50 kg/m^2	80 mm
Cockles (*Cerastoderma edule*)	7 – 16	20.0	50 kg/m^2	80 mm
Hard clam (*Mercenaria mercenaria*)	12 – 20	20.5	70 kg/m^2	80 mm
Native clam (*Ruditapes decussatus*)	12 – 20	20.5	50 kg/m^2	80 mm
Manila clam (*Ruditapes philippinarum*)	5 – 20	20.5	50 kg/m^2	–
Thick through shell (*Spisula solida*)	12	20.5	50 kg/m^2	–
Razor clams (*Ensis* spp.)	10	30.0	50 kg/m^2	Bundles of 12
Peppery furrow shell (*Scrobicularia plana*)	12	20.5	50 kg/m^2	–
Scallops (*Pecten maximus*)	10	35	–	–

- Lack of data.

Although depuration is effective in removing faecal bacterial contaminants (particularly *E. coli*) from bivalves, it is less effective or ineffective to remove other contaminants, such as norovirus, hepatitis A, naturally occurring marine vibrios (e.g. *V. parahaemolyticus* and *V. vulnificus*), toxins from HAB, toxic elements or organic chemicals (Lee *et al*., 2008a). In fact, several studies that have assessed the effects of depuration on levels of pathogenic microorganisms, toxic elements and total petroleum hydrocarbons (TPHs) in bivalve species, confirm the need for longer depuration times for pathogens, such as *Vibrio* spp and *Salmonella* spp., as well as for the majority of chemical contaminants, rather than the need of shorter depuration times for *E. coli* and fecal coliforms (Table 7).

Table 7. Examples of the effect of depuration on the elimination of microbiological and chemical contaminants from bivalves.

Species	Origin	Contaminant	Time (h)	Reduction (%)	Reference
Ruditapes decussatus (grooved carpet shell clam)	Timsah Lake, Ismilia (Egypt)	FC, *Vibrio* spp., fecal *Streptococcus*, coliphage	96	85, 50, 41, 69	El-Shenawy (2004)
		Fe, Ni, Co, Cu, Mn	48	47, 20, 28, 36	
Chamelea gallina (striped Venus clam)	Adriatic Sea adjacent to Cattolica (Italy)	FC	24	54	Maffei *et al.* (2009)
		E. coli	24	62	
		Salmonella spp.,	24	ND	
		V. parahaemolyticus			
		V. alginolyticus	24	NE	
	Adriatic Sea (Italy)	*E. coli*	24; 48	90; 99	Barile *et al.* (2009)
		Salmonella	24; 72-84	97.5; 99.9	
		Typhimurium			
		V. parahaemolyticus	24	75	
Paphia undulate (undulated surf clam)	Ismailia (Egypt)	*Vibrio* sp., *Shigella* sp.,	24	75, 31, 68, 36	El-Gamal (2011)
		E. coli, Salmonella sp.			
		Zn, Pb, Ni, Mn, Cu, Cr, Cd	24	44, 23, 25, 17, 61, 41, 75	
		TPHs	24; 72	90; 72	
Circe sinensis (clam)	Tolo Harbour (Hong Kong)	Cr, Ni, Zn	168	80, 25, 19	Cheung and Wong (1997)
Mytilus galloprovincialis (Mediterranean mussel)	Italy	*E. coli*, *V. cholerae* O1, *V. parahaemolyticus*	24	99.5, 92, 68	Croci *et al.* (2002)
	Italy	*E. coli*, TVC *V. parahaemolyticus, V. vulnificus*	24	99, 89, 97, 91	Cozzi *et al.* (2009)
	Adriatic Sea (Italy)	*E.coli*	6-12; 24	94; 99.5	Barile *et al.* (2009)
		Salmonella Typhimurium; V. parahaemolyticus	24; 36	99; 99.5	

Abbreviations: FC - faecal coliforms; ND – not detected; NE – not eliminated; TPHs - total petroleum hydrocarbons; TVC – total viable counts.

1.4.2 Transport

The transportation of live bivalve molluscs from harvesting areas to a distribution centre, depuration centre, relaying area or establishment should be done under salubrious conditions and accompanied by documentation for the identification of batches of live bivalve molluscs (Bettencourt *et al.*, 2012). After harvesting or treatment, food business operators storing and transporting live bivalve molluscs must ensure that they are kept at a temperature that does not adversely affect food safety or their viability. During all production process, food operators must use harvesting techniques and further handling that not cause additional contamination or damage to the animals, adequately protecting them from crushing, abrasion or vibration and not exposing to extreme temperatures or re-immersing them in water that could cause additional contamination (Lee *et al.*, 2008b).

Out of water transportation system is a preferred method for the shipment of live bivalves since they can survive relatively long periods out of water if they kept moist and cool, and the transport in seawater also requires larger volumes and space, increasing freight costs (Maguire *et al.*, 1999a; Dobson, 2001). The transport should not expose bivalves to contamination, crushing or extreme vibration in order to maintain the quality and safety of the product, as well as to avoid stress to specimens (Lee *et al.*, 2008a). The low temperature and high humidity should be controlled within the range 2–10 °C depending on species (Lee *et al.*, 2008a). Transport at extreme temperature conditions, for instance during the coldest winter and highest summer temperatures, are likely to be lethal (Dickie 1958; Strand and Brynjeldsen, 2003). But, certainly, the success of transport will depend upon the physiological characteristic of each organism when exposed to air, including its tolerability, air respiration capacity, and metabolism process (Ali *et al.*, 1999). The international trade, or even slow methods of transport for local markets, may result in potentially long periods between packing and arrival at the final destination and this will increase the difficulty in maintaining the optimum temperature during transport (Lee *et al.*, 2008a).

Although chilling is a technique commonly employed to retard bacterial growth and subsequent food spoilage, it is not appropriate for some bivalve species, such as the Sydney rock oyster (Jackson and Ogburn, 1999). This species is generally stored at ambient temperature prior to processing, avoiding temperature extremes, and once chilled this species will die within four days (Souness and Fleet, 1979). On the other hand, if bivalve species are transported at high temperatures the residual bacteria may multiply during this process, for example for *V. parahaemolyticus* and *V. vulnificus,* thus representing higher risks for consumers (Jackson and Ogburn, 1999). Consequently,

transport and storage of bivalves will be a compromise between the optimal conditions for bivalve survival and conditions that do not encourage bacterial growth and keep good physiological conditions (Jackson and Ogburn, 1999).

1.5 Stress physiology in bivalve molluscs

Capture method, handling procedures, aerial exposure, abrupt temperature changes, holding and transport of bivalves can result in varying degrees of stress, depending on the length of exposure and of species, which can in turn adversely affect their body condition (Cope and Waller, 1995). Therefore, the physiological status of bivalves can be measured by behavioural, biochemical/physiological and cellular tests/biomarkers, which can give a more complete picture of organisms' reaction to stress (Maguire *et al.*, 1999a; Joyner-Matos *et al.*, 2009). The behavioural tests commonly used are the valve closure (duration and frequency; known as gape) and burrowing rate (Lurman *et al.*, 2014). It is known that bivalve molluscs close entirely their valves and burrow directly beneath the sediment surface, especially when subjected to environmental stressors, such as high temperature and hypoxic conditions, in order to maintain oxygen levels (Strahl, 2011). The physiological tests include both non-invasive (e.g. survival rate, condition index and heart rate) and invasive processes (e.g. different breakdown products of adenosine triphosphate – ATP and glycogen content). In addition, the cellular-level biomarkers in bivalves typically measure molecular responses, which include the quantification of cellular damage and the expression of stress proteins, such as heat shock proteins and antioxidant enzymes.

Condition index (CI) has long been used for biological and commercial purposes (Baird, 1958) and it is also recognized as a useful biomarker reflecting the ability of bivalves to withstand adverse natural and/or anthropogenic stress (Mann, 1978). In industrial settings, CI has been adopted in international trade as a standard criterion to select the best product (Aníbal *et al.*, 2011). Hence, the CI may be considered as a measure of "fatness" and "marketability" of a commercially exploited species (Okumus and Stirling, 1998). Several equations have been proposed to calculate the CI of bivalves, indicating the relationship between one measurable body characteristics and one or several other body characteristics. For example, Maguire *et al.* (1999b) used the following equation:

$$CI = \frac{[total\ weight\ (g)]}{[shell\ lenght]\ \times\ [shell\ height] \times [\text{shell width}]\ (cm)} \times 10{,}000$$

K-value and adenylic energetic charge (AEC) value are indices that are calculated from the levels of ATP related compounds in relation to the effect of a short-term stress on the biochemistry and functional state of the animals by measuring the cell energy status (Raffin and Thebault, 1996; De Luca-Abbott *et al.*, 2000). K-value has been used as an index of quality that has received special attention for monitoring chemical assessment of the freshness in many species (Ehira and Uchiyama, 1987). This index is defined by Saito *et al.* (1959) as the ratio of the final breakdown products of ATP catabolism, namely inosine (HxR) and hypoxanthine (Hx), to the total amount of ATP and related compounds, i.e. adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), HxR and Hx:

$$K\ (\%) = \left[\frac{(Hx + HxR)}{(ATP + ADP + AMP + IMP + Hx + HxR)}\right] \times 100$$

Furthermore, AEC value is a linear measure of the ratio of ATP concentration to total adenylate concentration (Atkinson, 1968), which is quantified accordingly to the following equation:

$$AEC\ (\%) = \left[\frac{(ATP + 0.5ADP)}{(ATP + ADP + AMP)}\right] \times 100$$

Since all organisms use ATP for energy transduction, this adenylate cannot be stored, and any change in its concentration reflects an immediate modification in the capacity for energy production (Newsholme and Crabtree, 1986). This measure displays a wide range of values in invertebrates, that range between 0 and 1, according to the importance of the internal stress or to the variations in the external environment of the organisms (natural or anthropogenic; Atkinson, 1977; Picado and Le Gal, 1999). High AEC values (0.8-0.9) tend to occur in organisms found in non-limiting environments, while values between 0.5 and 0.7 indicate organisms suffering sub-optimal conditions, and animals with AEC lower than 0.5 do not to have the capacity to recover from the associated stress (Bayne *et al.*, 1985). Therefore, an animal suffering more stress will use more energy to counteract the stress, thus lowering its AEC level (Maguire *et al.*, 1999b). In terms of nutritional reserves, carbohydrates play an important role in bivalves' energy production and gametogenesis (Mathieu and Lubet, 1993), with glycogen representing the most prominent carbohydrate stored in marine bivalves (Hummel *et al.*, 1989). Commonly used to assess the physiological condition of bivalves, glycogen content can indicate stressful environmental

conditions (Patterson *et al.*, 1997). Moreover, reduction or depletion in metabolic reserves such as glycogen is often associated with mass mortality of adult bivalves (Barber and Blake, 1981; Robert *et al.*, 1993).

1.5.1 Thermal tolerance limits and metabolism

Temperature is one the most important environmental factors affecting molecules kinetic energy, biochemical reactions and physiological processes in marine ectothermic organisms (e.g. bivalve molluscs; Somero, 1969; Mora and Ospína, 2001). Under a global change context, it is expected that the average global sea surface temperatures will increase 0.3–4.8 °C by the end of the 21st century due to climate change (IPCC, 2013) and the frequency and severity of extreme high temperature events (heat waves) will increase over the next decade (IPCC, 2013). As a consequence, this thermal challenge will lead to potentially negative impacts in performance, survival and distribution limits of marine organisms (Pörtner, 2010; Rosa *et al.*, 2012, 2013, 2014; Vasseur *et al.*, 2014), particularly those that already live close to their thermal tolerance limits (Stillman and Somero, 2000; Helmuth *et al.*, 2006; Hoegh-Guldberg *et al.*, 2007; Tewksbury *et al.*, 2008). Therefore, physiological thermal tolerance limits are often directly linked to current distribution range shifts of marine organisms, reflecting the existence of latitudinal thermal gradients (Pörtner and Knust, 2007; Somero, 2010). It has been suggested that species with higher thermal tolerances will be better able to cope with global warming (Calosi *et al.*, 2008) or, conversely, that more warm-adapted species will be at a disadvantage because they tend to live closer to their absolute tolerance limits (Stillman and Somero, 2000; Compton *et al.*, 2007; Somero, 2010; Sorte *et al.*, 2011) and have lower acclimation potentials (Somero, 2010).

The knowledge of thermal tolerance is the first step to understand how vulnerable species are (Rivadeneira and Fernández, 2005). Fitness is a measure used to understand how organisms respond to environmental variation (Miller and Stillman, 2012). The organisms tend to maximize their fitness at an “optimal” environmental range, and can survive only short periods in environmental conditions that exceed a threshold in their “critical tolerance limits” (Miller and Stillman, 2012). Critical limits may serve to define species distributions, community structure, and how communities respond to environmental changes (Miller and Stillman, 2012). Variation in fitness between environmental factors, such as temperature, that define an organism's critical limits can be measured as a “performance curve” (Figure 9) (Pörtner *et al.*, 2006; Angilletta, 2009).

Performance is maximized under a range of optimal temperatures, and declines to zero just beyond upper and lower critical temperatures (CTMax and CTMin) that define the organism's thermal tolerance range (Miller and Stillman, 2012). Eurythermal organisms, like bivalve molluscs, have a wide thermal breadth, and can inhabit a wide range of thermal environments, in contrast with stenothermal organisms (Miller and Stillman, 2012). As temperatures increase or decrease from the optimal, performance is reduced. The temperatures where performance begins to decline are referred to as pejus temperatures, and are less extreme than the critical limits (Figure 9). Pejus temperatures (the latin "pejus" means getting worse) may represent thermal transitions that are more ecologically relevant than critical thermal limits, because well before reaching temperatures that result in imminent death, an organism may be required to mount compensatory mechanisms (Pörtner *et al.*, 2006).

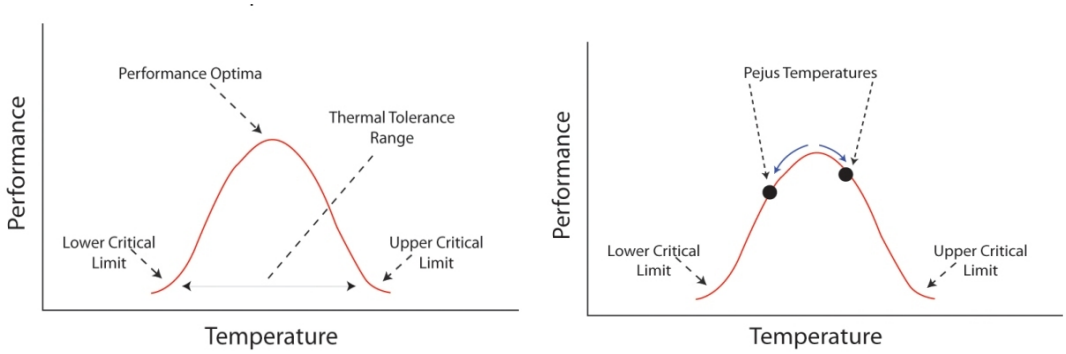


Figure 9. Hypothetical physiological performance curves for ectothermic animals. Critical limits refer to temperatures beyond which only short-term exposure is possible. (Pörtner *et al.*, 2006; Miller and Stillman, 2012).

At the physiological level, oxygen availability can limit aerobic metabolism and consequently thermal tolerance (Frederich and Pörtner, 2000; Pörtner *et al.*, 2004; Melzner *et al.*, 2007; Pörtner and Knust, 2007; Rosa and Seibel, 2008; Rosa *et al.*, 2014). Ocean warming may decrease oxygen availability and increase the metabolic rates of organisms, leading to a decline in aerobic performance, with a progressive transition towards an anaerobic mode of energy production [principles of the concept of "oxygen- and capacity-limitation of thermal tolerance" (Pörtner *et al.*, 2004; Pörtner and Knust, 2007)]. Thus, the reduction in aerobic scope of ectotherms with global warming is assumed not to be caused by lower levels of ambient oxygen, but through limited capacity of oxygen supply mechanisms (ventilatory and circulatory systems), in order to meet an animal's temperature-dependent oxygen demand (Pörtner and Knust, 2007). As consequence, the survival, reproduction and recruitment of marine ecthoterms will be in

jeopardy by warming (Mora and Ospína, 2001). At the behavioral level, changes in reproductive strategies and life history patterns might also occur (Angilletta *et al.*, 2006).

A common response of most bivalves to an environmental stressor such as warming includes valve closure (with isolation of tissues) and burrowing into the sediment (Vinu Chandran, 2002; Ortmann and Grieshaber, 2003). This behavioural tactic restricts gas exchange, reduces aerobic metabolism, and may be an important strategy for longer survival under unfavourable conditions (Anestis *et al.*, 2010). Some authors suggested that bivalves become anaerobic after a short period of valve closure, even without ambient stressors (Sobral and Widdows, 1997; Ortmann and Grieshaber, 2003). The great reduction in the bivalves' metabolism (e.g. 90% in Asian clam *Corbicula fluminea*) after closing the valves saves a great amount of energy compared to the tenfold higher metabolic rate when the valves are open (Ortmann and Grieshaber, 2003). Previous studies have indicated that metabolic depression is usually accompanied by valve closure in bivalves (Ortmann and Grieshaber, 2003; Anestis *et al.*, 2007) and has frequently been described among invertebrates facing severe stress (Guppy and Withers, 1999; Anestis *et al.*, 2007). However, the ability to close the valves and thus protecting the animal from any major environmental stress, such as predators, water pollution or contamination of the gills, along with the concomitant reduction in the metabolic rate in an aerobically respiring animal, saves an enormous amount of energy and can be particularly beneficial during periods of starvation as well as if an animal has to rely on inefficient anaerobic pathways (Grieshaber *et al.*, 1994; Ortmann and Grieshaber, 2003).

1.5.2 Differences between alien invasive and native species

Alien invasive species (AIS), also known as exotic, introduced or non-native species, are those introduced, intentionally or accidentally, by human activity to a new geographic area or ecosystem outside of its natural distribution range, being a threaten to the ecosystems, habitats and/or other species (GISP, 2008). AIS are causing an array of ecological (e.g. impact on biodiversity, ecosystem structure and function), economic and health impacts for invaded countries (Mack *et al.*, 2000; Hellmann *et al.*, 2008; Simberloff *et al.*, 2012), that may become visible only long after introduction (Essl *et al.*, 2011). Clams and oysters (e.g. Manila clam *R. philippinarum* and American oyster *C. gigas*) are considered the best-known examples of negative impacts of AIS in European coastal areas in general and in Mediterranean in particular (e.g. in the lagoon of Venice; Ambrogi, 2000). The high potential for dispersal, fast growth, high reproductive rates and plasticity (ability to adjust traits to match different conditions) have been associated with AIS, which will

have a major impact on local benthic fauna and flora, since these species competes for food and space with other filter-feeding invertebrates (Otero *et al.*, 2013). Many introduced species also become invasive after persisting for a long time at low abundances in the introduced habitat (Mack *et al.*, 2000; Sakai *et al.*, 2001), possibly after evolutionary adjustments to the novel environments (Mooney and Cleland, 2001).

Particularly, higher temperature can be a boom for AIS in marine ecosystems, because induce inter-specific competitions, where the most warm-adapted species will replace the native species (Stachowicz *et al.*, 2002). This finding is corroborated in several studies conducted with AIS, such as the mussel *M. galloprovincialis* (Lockwood and Somero, 2011), the clam *C. fluminea* (Weitere *et al.*, 2009) and marine fouling community (namely several bryozoan and tunicate species; Sorte *et al.*, 2010). AIS are known to be more eurythermal (ability to maintain physiological function over a wide range of temperatures) and have larger latitudinal ranges than native species, which is indicative of their ability to tolerate a broader range of environmental conditions and their potential for greater success at higher temperatures (Figure 10; Dukes and Mooney, 1999). This higher thermal tolerance of AIS is likely due in part to its enhanced ability to induce changes in the expression of particular genes and proteins in response to acute heat stress (Lockwood and Somero, 2011). In contrast, the native species usually display lower thermal tolerance limits and, consequently, are unable to physiologically respond to extreme conditions (Calosi *et al.*, 2008; Somero, 2010).

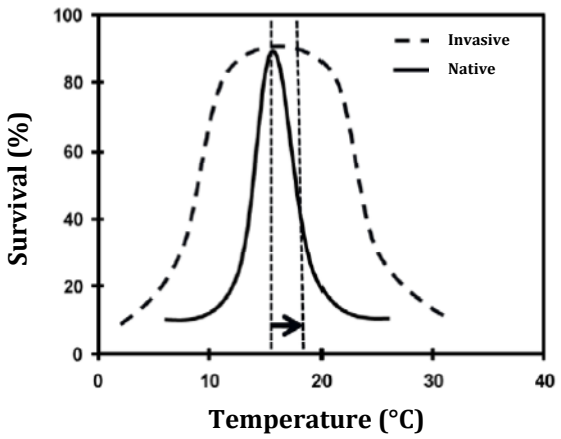


Figure 10. Conceptual model of greater invasive eurythermality. If an invasive (dashed line) has a broader range or higher limit of temperature tolerance than a native (solid line) then an increase in temperature (illustrated by a shift from the left to the right vertical dashed line) is likely to have a disproportionately negative impact on the native species. This physiological mechanism has the potential to precipitate changes in community composition, with increases and decreases in invasive and native relative abundance, respectively (Zerebecki and Sorte, 2011).

Currently, there are several studies assessing physiological, biochemical and molecular responses to environmental stress between AIS and native species. Indeed, the success of AIS species can be explained by the following responses to a determined environmental stress (e.g. temperature): higher fitness, survival and growth rates; lower activities of enzymes involved in ATP generation (malate dehydrogenase – MDH and citrate synthase – CS); lower metabolism (oxygen consumption rates); lower cardiac physiology (resting heart rate and ventilatory rate); higher thermal tolerance of cardiac function (critical heart rate); higher expression of particular genes and proteins (heat shock proteins – HSP24 and HSP70); and higher levels of glycogen reserves, but lower protein and total lipids contents; Table 8; Hofmann and Somero, 1996; Stachowicz *et al.*, 2002; Schneider, 2008; Lockwood and Somero, 2011; Maazouzi *et al.*, 2011; Zerebecki and Sorte, 2011;). These disparate thermal physiologies of both species are likely determinants of their distinct biogeographical distributions, being invasive species generally more warm-adapted than the native ones.

Table 8. Physiological responses of native and alien invasive species (AIS) to temperature increase.

Alien invasive species (AIS)	Native species	Physiological parameter	Physiological response of AIS	Reference
Mytilus galloprovincialis (mussel)	*Mytilus trossulus* (mussel)	MDH and CS activities	lower	Lockwood and Somero (2011)
		resting heart rate	lower	
		critical heart rate	higher	
		HSP24 expression	higher	
		HSP70 induction	higher	Hofmann and Somero (1996)
		survival and growth rates	higher	Schneider (2008)
Diplosoma listerianum (tunicate)	*Distaplia occidentalis* (tunicate)	HSP70 levels	higher	Zerebecki and Sorte (2011)
Botrylloides violaceous and *Diplosoma listerianum* (ascidian)	*Botryllus schlosseri* (ascidian)	growth rate	higher	Stachowicz *et al.* (2002)
		fitness	higher	
Dikerogammarus villosus (crustacean)	*Gammarus pulex* (crustacean)	survival and locomotory activity	lower	Maazouzi *et al.* (2011)
		oxygen consumption and ventilatory rates	lower	
		glycogen	higher	
		protein	lower	
		total lipids	similar	

Some authors also argue that the differences found in enzymatic thermal sensitivity could result in a higher heat tolerance of energy-producing metabolic reactions in the invasive species, thus sustaining ATP production at elevated temperatures to support functions like muscle contraction (Lockwood and Somero, 2011). In addition to physiological abilities,

other mechanisms also may contribute significantly to the successful invasion of a species, including the life-history strategies (e.g., reproductive potential, body size), adaptations to certain habitats, and release from predators and diseases in invaded communities (Rejmanek, 1996; Calvo-Ugarteburu and McQuaid, 1998; Radford and Cousens, 2000). Overall, these studies are very useful to understand the physiological mechanisms that lead to the greater success of invasive species rather than the native ones.

1.5.3 Oxidative stress biomarkers

Oxidative stress is a common consequence of the metabolic and acid–base disturbance in marine organisms, being usually implicated in physiological responses to a wide variety of environmental stressors, such as elevated temperature, ocean acidification and anthropogenic contamination (e.g. PCBs, PAHs, phenols and toxic elements; Matoo *et al.*, 2013). Presently, a large amount of information is available concerning oxidative stress in marine invertebrates subjected to such stressors, particularly in bivalves (e.g. Abele *et al.*, 2001, 2002; Bergayou *et al.*, 2009; Matoo *et al.*, 2013). An oxidative stress response occurs when there is a mismatch between generation and detoxification of reactive oxygen species (ROS) (Abele *et al.*, 2001, 2002). ROS are chemically reactive molecules derived from the normal metabolism of oxygen and play important roles in cell signalling and homeostasis (Cadenas, 1989). There are many types of ROS including singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO\cdot$), with the latter one being the most reactive and destructive (Pannunzio and Storey, 1998; Lesser, 2006). Generally, ROS are not harmful to the organism, but during periods of increased environmental stress, ROS can increase dramatically causing injury to cells, namely in lipids, proteins and DNA, inhibiting physiological processes (Gerschman *et al.*, 1954; Cadenas, 1989; Mittler, 2002). Beyond the ROS production, the elevations in antioxidant defences and the occurrence of oxidative cellular damage are the measurements generally used in oxidative stress (Winston and Di Guilio, 1991).

The most frequent cellular injury caused by ROS is lipid peroxidation, a self-propagating process in which a peroxyl radical is formed when a ROS has sufficient reactivity to abstract a hydrogen atom from an intact lipid (Halliwell and Gutteridge, 2007). Lipid peroxidation consists of four phases, namely initiation, propagation, decomposition and termination and is usually quantified via the assessment of a specific end-product of the oxidative degradation process of lipids, the malondialdehyde (MDA) levels (Uchiyama and Mihara, 1978; Gutteridge and Halliwell, 1990; Pannunzio and Storey, 1998). Furthermore, ROS may indirectly damage DNA via the production of oxidized lipid and protein by-

products that form adducts (Møller and Wallin, 1998). Proteins represent a third major biomolecule in cells susceptible to ROS attack, but measurements of oxidative damage to proteins (i.e., protein carbonyl groups) have only been investigated in a few bivalve species (Kirchin *et al.*, 1992; Walker *et al.*, 2000).

Marine organisms have also a variety of defences, known as antioxidants to combat harmful ROS and stave off oxidation and subsequent damage (Lesser, 2006). Antioxidants prevent free radical production, scavenge existing ROS and unpaired electrons and purge chain reactions, and as such they are considered primary defences against oxidative stress (Gould, 2003). Thus, the term antioxidant applies to "any substance that significantly delays or inhibits oxidation" (Halliwell and Gutteridge, 2007), and incorporates enzymes, reductants and vitamins (e.g. α-tocopherol) (Kappus, 1987). The antioxidant enzymes that play an important role in oxidative stress prevention are: (i) superoxide dismutase (SOD), which converts $O_2^{\bullet-}$ in H_2O_2, (ii) catalase (CAT), which removes H_2O_2 converting to H_2O and O_2 in order to avoid its accumulation in cells and tissues, (iii) glutathione reductase (GR), which furnishes cells with the antioxidant glutathione (GSH, i.e. by reacting with $O_2^{\bullet-}$ and $OH^{\bullet}$) and further supplies the enzyme activity of glutathione peroxidise (GPX) and glutathione-S-transferase (GST), (iv) GPX, which also eliminates H_2O_2 using GSH as substrate, and (v) GST, which, in association with GSH, transforms xenobiotics into other conjugates as part of a detoxification route (Lesser, 2006). When ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops (Sies, 1991).

1.5.4 Heat shock response

A heat shock response (HSR) can be induced by marine organisms under environmental stress, such as thermal stress, that is characterized by the synthesis of heat-shock proteins (HSPs) in order to promote refolding of denatured proteins and prevent further protein unfolding and aggregation (Morimoto and Santoro, 1998; Feder and Hofmann, 1999). HSPs are a family of highly conserved molecular chaperones that are either constitutively expressed (e.g., heat shock cognate 70 [HSC70]) or induced upon changes in temperature or other stressors (e.g., heat shock protein 70 [HSP70]) (Morimoto and Milarski, 1990; Ciavarra *et al.*, 1994), and are expressed in a tissue-specific manner (Yamashita *et al.*, 2010). HSP70 can be elevated several hundred times compared to non-stress conditions, while HSC70 is constitutively expressed in normal cells and moderately induced (only several folds) under normal growth conditions (Ali *et al.*, 1996; Chuang *et al.*, 2007). In addition, the interplay between oxidative stress, antioxidants and HSPs is thus extremely important for the survival to critical temperature stress (Heise *et al.*, 2006). These

oxidative stress parameters have been widely used as biomarkers to monitor the impact of environmental factors on several animal species. Currently, there is a special interest to study the impact of temperature on the oxidative stress of marine biota, namely fish (Heise *et al.*, 2006), crustaceans (Liberato *et al.*, 2003) and bivalves, such as *C. virginica, M. mercenaria* (Matoo *et al.*, 2013), *Yoldia eightsi* (Abele *et al.*, 2001) and *R. philippinarum* (Tamayo *et al.*, 2013).

1.6 General aims and thesis outline

This thesis is based on a set of experiments carried out in Guia Marine Laboratory (Center of Oceanography, Faculty of Sciences, University of Lisbon) and the Portuguese Institute for the Sea and Atmosphere (IPMA). The main objective of this thesis was to investigate different biological issues related with bivalve molluscs, particularly investigating the bivalve consumption behaviour, evaluating the microbiological, chemical and physiological responses of native and invasive clams from Tagus estuary (recently classified as being in the B category harvesting area) to depuration and transport, as well as the impact of environmental warming in metabolism, oxidative stress and nutritional quality of clam species. The thesis is composed by nine chapters and includes seven scientific papers published or *in press* in peer-reviewed international journals, each corresponding to a chapter (2 to 8). Finally, the main objectives of each chapter are presented below.

In Chapter 1 is presented a general introduction about bivalves, including their importance and trade chain in Portugal. Additionally, the main benefits and risks associated with bivalve consumption are described, as well as the stress physiology of bivalves.

Since bivalves are economically and nutritionally very important for human consumption, playing a central role in the Portuguese gastronomy, an online survey was performed to understand Portuguese consumers' attitudes and perceptions of bivalve molluscs according to their demographic and socio-economic status (Chapter 2). The results of this chapter provide a useful knowledge of consumption profile and the risks associated to bivalve consumption.

Once identified the most important species from Tagus estuary, such as *Venerupis pullastra* (native clam) and *Ruditapes philippinarum* (alien invasive clam), a one-year sampling was performed in Tagus estuary (Portugal) to examine the influence of seasonal and environmental parameters on the occurrence of several bacteria (total viable counts –

TVC, *E. coli*, *Salmonella* spp. and *Vibrio* spp.) in both species and correlate with levels found in water and sediment in order to identify the main contamination source (Chapter 3).

In Chapters 4 and 5, experiments were designed to characterize and understand the microbiological and physiological responses of both clam species in two critical steps of the trade chain, i.e. depuration and subsequent transport under simulated conditions (at 4 and 22 °C). The results are discussed regarding the effectiveness of depuration in reducing bacterial load and the confirmation of the best transport temperature and duration taking into account low bacteria levels, good physiological conditions and high quality of bivalves.

Chapter 6 presents the effects of depuration on the potential accumulation/removal of toxic elements (Hg, Cd, Pb and As) and macro/trace elements (S, Cl, K, Ca, Fe, Zn, Br, Cu, Se, Rb and Sr) in three economically and ecologically important bivalve species (*R. philippinarum*, *Mytilus galloprovincialis* and *Scrobicularia plana*) from Tagus estuary. Additionally, the animal condition of these species (survival, condition index and glycogen content) was also investigated during eight days of depuration.

Since a future ocean warming is expected to directly impact the performance and survival of marine organisms, an experiment was performed to investigate the physiological and biochemical responses, as well as the nutritional quality, of native (*Ruditapes decussatus*) and alien invasive (*R. philippinarum*) clams to thermal stress (Chapters 7 and 8). Results of this chapter are discussed, evaluating the effect of temperature on metabolism and oxidative status of clams through the determination of routine metabolic rates and several activities of antioxidant enzymes (Chapter 7). In addition, the effect of thermal stress on nutritional quality of both species was also evaluated through the determination of protein, glycogen and fatty acids contents (Chapter 8). It is noteworthy that in this last set of experiments (Chapters 7 and 8), other clam species (*R. decussatus*) was selected in order to compare two species belonging to the same genus and also due the recent overexploitation of *V. pullastra* in Tagus estuary.

Finally, a general discussion of the highlights obtained in the previous chapters is presented in Chapter 9. This chapter also point out the main conclusions within the context of the microbiological risk of clams' consumption as well as their physiological, microbiological and chemical responses during depuration and transport and the effect of thermal stress on physiology/biochemistry and nutritional quality of clams' species. Additionally, in this chapter is also revealed the research perspectives inherent to the subject and related research areas.

1.7 References

Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S. 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *Journal of Experimental Biology,* 205, 1831–1841.

Abele, D., Tesch, C., Wencke, P., Pörtner, H.O. 2001. How does oxidative stress relate to thermal tolerance in the Antarctic bivalve *Yoldia eightsi*?. *Antarctic Science,* 13(2), 111–118.

Alam, M.J., Tomochika, K.I., Miyoshi, S.I., Shinoda, S. 2002. Environmental investigation of potentially pathogenic *Vibrio parahaemolyticus* in the Seto-Inland Sea, Japan. *FEMS Microbiology Letters,* 208, 83–87.

Ali, A., Salter-Cid, L., Flajnik, M.F., Heikkila, J.J. 1996. Isolation and characterization of a cDNA encoding a *Xenopus* 70-kDa heat shock cognate protein, Hsc70. *Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology,* 113, 681–687.

Ali, F., Nakamura, K., Yamasaki, S. 1999. Physiological characteristics of aerial survival in Japanese clam, *Ruditapes philippinarum* (Adams and Reeve). *Suisan zoshoku,* 47(3), 381–389.

Ambrogi, A.O. 2000. Biotic invasions in a Mediterranean lagoon. *Biological Invasions,* 2(2), 165–176.

Anacleto, P., Barrento, S., Nunes, M.L., Rosa, R., Marques, A. 2014. Portuguese consumers' attitudes and perceptions of bivalve molluscs. *Food Control,* 41, 168–177.

Anestis, A., Lazou, A., Pörtner, H.O., Michaelidis, B. 2007. Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology,* 293(2), R911–R921.

Anestis, A., Pörtner, H.O., Karagiannis, D., Angelidis, P., Staikou, A., Michaelidis, B. 2010. Response of *Mytilus galloprovincialis* (L.) to increasing seawater temperature and to marteliosis: metabolic and physiological parameters. *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology,* 156(1), 57–66.

Angilletta, M.J. 2009. Thermal adaptation, a theoretical and empirical synthesis. Oxford University Press, Oxford, UK.

Angilletta, M.J., Oufiero, C.E., Leaché, A.D. 2006. Direct and indirect effects of environmental temperature on the evolution of reproductive strategies: an information-theoretic approach. *American Naturalist,* 168(4), E123-E135.

Aníbal, J., Esteves, E., Rocha, C. 2011. Seasonal variations in gross biochemical composition, percent edibility, and condition index of the clam *Ruditapes decussatus* cultivated in the Ria Formosa (South Portugal). *Journal of Shellfish Research,* 30(1), 17–23.

Anonymous. 2012. *Community guide to the principles of good practice for the microbiological classification and monitoring of bivalve mollusc production and relaying areas with regard to Regulation 854/2004,* 35 pp.

Arias, C.R., Macian, M.C., Azar, R., Garay, E., Pujalte, M.J. 1999. Low incidence of *Vibrio vulnificus* among *Vibrio* isolates from sea water and shellfish of the western Mediterranean coast. *Journal of Applied Microbiology,* 86, 125–134.

Atkinson, D.E. 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry,* 7, 4030–4034.

Atkinson, D.E. 1977. *Cellular energy metabolism and its regulation.* Academic Press, New York.

Baird, R.H. 1958. Measurement of condition in mussels and oysters. *Journal du Conseil Permanent International pour L'Exploration de la Mer,* 23(2), 249–257.

Bandarra, N.M., Calhau, M.A., Oliveira, L., Ramos, M., Dias, M.G., Bártolo, H., Faria, M.R., Fonseca, M.C., Gonçalves, J., Batista, I., Nunes, M.L. 2004. Composição e valor nutricional dos produtos da pesca mais consumidos em Portugal. *Publicações avulsas do IPIMAR*, 11, 103 pp.

Barber, B.J., Blake, N.J. 1981. Energy storage and utilization in relation to gametogenesis in *Argopecten irradians concentricus* (Say). *Journal of Experimental Marine Biology and Ecology,* 52, 121–134.

Barbieri, E., Falzano, L., Fiorentini, C., Pianetti, A., Baffone, W., Fabbri, A., Matarrese, P., Casiere, A., Katouli, M., Kuhn, I., Molby, R., Bruscolini, F., Donelli, G. 1999. Occurrence, diversity and pathogenecity of halophilic *Vibrio* spp. a non-O1 *Vibrio cholerae* from estuarine waters along the Italian Adriatic coast. *Applied and Environmental Microbiology,* 65, 2748–2753.

Barile, N.B., Scopa, M., Nerone, E., Mascilongo, G., Recchi, S., Cappabianca, S., Antonetti, L., 2009. Study of the efficacy of closed cycle depuration system on bivalve molluscs. *Veterinaria Italiana,* 45(4), 555–566.

Bayne, B.L., Brown, D.A., Burns, K., Dixon, D.R., Ivanovici, A., Livingstone, D.R., Lowe, D.M., Moore, M.N., Stebbing, A.R.D., Widdows, J. 1985. *The effects of stress and pollution on marine animals.* Praeger Scientific, New York, 384 pp.

Bergayou, H., Mouneyrac, C., Pellerin, J., Moukrim, A. 2009. Oxidative stress responses in bivalves (*Scrobicularia plana*, *Cerastoderma edule*) from the Oued Souss estuary (Morocco). *Ecotoxicology and Environmental Safety,* 72, 765–769.

Bettencourt, F., Matias, D., Soares, F. 2012. Microbiological monitoring of bivalve molluscs growing areas: a food safety and public health approach. *Aquaculture Europe,* 37(3), 11–15.

Brands, D.A., Inman, A.E., Gerba, C.P., Maré, C.J., Billington, S.J., Saif, L.A., Levine, J.F., Joens, L.A. 2005. Prevalence of *Salmonella* spp. in oysters in the United States. *Applied and Environmental Microbiology,* 71(2), 893-7.

Burkhardt, W. III, Calci, K.R. 2000. Selective accumulation may account for shellfish associated viral illness. *Applied and Environmental Microbiology,* 66(4), 1375–1378.

Burkhardt, W. III, Watkins, W.D., Rippey, S.R. 1992. Seasonal effects on accumulation of microbial indicator organisms by *Mercenaria mercenaria*. *Applied and Environmental Microbiology,* 58(3), 826–831.

Butt, A.A., Aldridge, K.E., Sanders, C.V. 2004. Infections related to the ingestion of seafood part I: viral and bacterial infections. *The Lancet Infectious Diseases,* 4(4), 201–212.

Cabelli, V.J., Heffernan, W.P. 1970. Accumulation of *Escherichia coli* by the Northern Quahaug. *Applied Microbiology,* 19(2), 239–244.

Cadenas, E. 1989. Biochemistry of oxygen toxicity. *Annual Review of Biochemistry,* 58, 79–110.

Calosi, P., Bilton, D.T., Spicer, J.I. 2008. Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biology Letters,* 4, 99–102.

Calvo-Ugarteburu, G., McQuaid, C.D. 1998. Parasitism and invasive species: effects of digenetic trematodes on mussels. *Marine Ecology Progress Series,* 160, 149–163.

Cefas. 2010. *Cefas protocol for inspection and approval of purification (depuration) systems: England and Wales.* Available at http://www.cefas.defra.gov.uk/media/540968/purification generic protocol_v8 final.pdf. Accessed 26 May 2014.

Censi, P., Spoto, S.E., Saiano, F., Sprovieri, M., Mazzola, S., Nardone, G., Di Geronimo, S.I., Punturo, R., Ottonello, D. 2006. Heavy metals in coastal water systems. A case study from the northwestern Gulf of Thailand. *Chemosphere,* 64, 1167–1176.

Cheung, Y.H., Wong, M.H. 1997. Depuration and bioaccumulation of heavy metals by clams from tolo harbour, Hong Kong. *Toxicological & Environmental Chemistry,* 58(1–4), 103–116.

Chuang, K.-H., Ho, S.-H., Song, Y.-L. 2007. Cloning and expression analysis of heat shock cognate 70 gene promoter in tiger shrimp (*Penaeus monodon*). *Gene,* 405, 10–18.

Ciavarra, R.P., Goldman, C., Wen, K.-K., Tedeschi, B., Castora, F.J. 1994. Heat stress induces hsc70/nuclear topoisomerase I complex formation in vivo: evidence for hsc70-mediated, ATP-independent reactivation in vitro. *Proceedings of the National Academy of Sciences USA,* 91(5), 1751–1755.

Coles, J.A., Pipe, R.K. 1994. Phenoloxidase activity in the hemolymph and hemocytes of the marine mussel *Mytilus edulis*. *Fish & Shellfish Immunology,* 4, 337–352.

Comps, M., Bonam, J.R., Vago, C., Campillo, A. 1976. Une virose de l'huitre portugaise (*Crassostrea angulata* LMK). *Comptes rendus de l'Académie des Sciences, Paris (Ser D),* 282, 1991–1993.

Compton, T.J., Rijkenberg, M.J.A., Drent, J., Plersma, T. 2007. Thermal tolerance ranges and climate variability a comparison between bivalves from differing climates. *Journal of Experimental Marine Biology and Ecology,* 352, 200–211.

Cope, G.W., Waller, D.L. 1995. Evaluation of freshwater mussel relocation as a conservation and management strategy. *Regulated Rivers: Research and Management,* 11(2), 147–155.

Cozzi, L., Suffredini, E., Ciccaglioni, G., Croci, L. 2009. Depuration treatment of mussels experimentally contaminated with *V. parahaemolyticus* and *V. vulnificus*. *In: Proceeding ICMSS09*. Nantes, France, 6 pp.

Croci, L., Suffredini, E., Cozzi, L., Toti, L. 2002. Effects of depuration of molluscs experimentally contaminated with *Escherichia coli*, *Vibrio cholerae* O1 and *Vibrio parahaemolyticus*. *Journal of Applied Microbiology,* 92(3), 460–465.

De Luca-Abbott, S., Creese, R.G., Lewis, G.D., Wells, R.M.G. 2000. Adenylate energy charge and total adenylate nucleotide pool as biomarkers of sublethal stress in the cockle, *Austrovenus stutchburyi*. *Australasian Journal of Ecotoxicology,* 6, 35–44.

Derolez, V., Soudant, D., Fiandrino, A., Cesmat, L., Serais, O. 2013. Impact of weather conditions on *Escherichia coli* accumulation in oysters of the Thau lagoon (the Mediterranean, France). *Journal of Applied Microbiology,* 114(2), 516–525.

DGPA (Direcção-Geral das Pescas e Aquicultura). 2010. *Centros de depuração e de expedição de moluscos bivalves vivos (licenciados e com nº de aprovação).* Direção-Geral das Pescas e Aquicultura, 9 pp.

Dickie, L.M. 1958. Effects of high temperature on survival of the giant scallop. *Journal of the Fisheries Research Board of Canada,* 15, 1189–1211.

Dionísio, L.P., João, M., Ferreiro, V., Fidalgo, M.L., Rosado, M.E., Borrego, J.J. 2000. Occurrence of *Salmonella* spp. in estuarine and coastal waters of Portugal. *Antonie van Leeuwenhoek,* 78, 99–106.

Dobson, G. 2001. An improved method of packing to minimise mortality in juvenile trochus during transport. *SPC Trochus Information Bulletin,* 8, 22–23.

Dong, F.M. 2001. *The nutritional value of shellfish.* Available at http://www.wsg.washington.edu/communications/online/shellfishnutrition_09.pdf Accessed 26 March 2014.

DTU (Technical University of Denmark). 2014. *Danish Food Composition Databank.* National Food Institute. Available at http://www.foodcomp.dk/v7/fcdb_grpsearchres.asp?MainGrp=08. Accessed 08 May 2014.

Dukes, J.S., Mooney, H.A. 1999. Does global change increase the success of biological invaders?. *Trends in Ecology and Evolution,* 14, 135–139.

Dutka, B.J. 1973 Coliforms are an inadequate index of water quality. *Journal of Environmental Health,* 36(11), 39–46.

EC (European Commission). 2001. *Opinion of the Scientific Committee on Veterinary Measures relating to Public Health on Vibrio vulnificus and Vibrio parahaemolyticus (in raw and undercooked seafood).* European Commission Health & Consumer Protection Directorate General. Directorate C—Scientific Opinions, Brussels, Belgium, pp. 1–64.

EC (European Commission). 2002. *Opinion of the scientific committee on food on the risks to human health of polycyclic aromatic hydrocarbons in food.* SCF/CS/CNTM/PAH/29 Final, Brussels, 84 pp.

EC (European Commission). 2004a. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for food of animal origin*. Official Journal of the European Union,* L226, 22–82.

EC (European Commission). 2004b. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal of the European Union,* L226, 83–127.

EC (European Commission). 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006. Setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union,* L364, 5–24.

EC (European Commission). 2007. Commission Regulation (EC) 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union,* L322, 1–18.

Efstratiou, M.A., Tsirtsis, G. 2009. Do 2006/7/EC European union bathing water standards exclude the risk of contact with *Salmonella* or *Candida albicans*?. *Marine Pollution Bulletin,* 58(7), 1039–1044.

Ehira, S., Uchiyama, H. 1987. Determination of fish freshness using the K-value and comments on some other biochemical changes in relation to freshness. *In:* Kramer, D.E., Liston, J. (Eds.), *Seafood quality determination.* Elsevier Science, New York, pp. 185–207.

El-Gamal, M.M. 2011. The effect of depuration on heavy metals, petroleum hydrocarbons, and microbial contamination levels in *Paphia undulata* (Bivalvia: Veneridae). *Czech Journal of Animal Science,* 56(8), 345–354.

El-Shenawy, N.S. 2004. Heavy metal and microbial depuration of the clam *Ruditapes decussatus* and its effect on bivalve behavior and physiology. *Environmental Toxicology,* 19, 143–153.

Essl, F., Dullinger, S., Rabitsch, W., Hulme, P.E., Hülber, K., Jarošík, V., Kleinbauer, I., Krausmann, F., Kühn, I., Nentwig, W., Vilà, M., Genovesi, P., Gherardi, F., Desprez-Loustau, M.-L., Roques, A., Pyšek, P. 2011. Socioeconomic legacy yields an invasion debt. *Proceedings of the National Academy of Sciences of the United States of America,* 108, 203–207.

EUROSTAT. 2014. *Statistics.* Available at http://epp.eurostat.ec.europa.eu/portal/page/portal/statistics/themes. Accessed 12 February 2014.

FAO (Food and Agriculture Organization of the United Nations). 2014. *Statistics.* Available at http://www.fao.org/fishery/statistics/en. Accessed 12 February 2014.

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization). 2001. *Hazard identification, exposure assessment and hazard characterization of Vibrio spp. in seafood.* FAO/WHO, MRA 01/03, Rome, Italy.

FAO/WHO (Food and Agriculture Organization/World Health Organization). 2002. *Risk assessment of Campylobacter spp. in broiler chickens and Vibrio spp. in seafood.* Report of a Joint FAO/WHO Expert Consultation, FAO Food and Nutrition 75, FAO/WHO, Bangkok, Thailand, 59 pp.

Feder, M.E., Hofmann, G.E. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology,* 61, 243–282.

Feng, P., Weagant, S.D., Grant, M.A. 2002. Enumeration of *Escherichia coli* and the coliform bacteria. *In:* Bacteriological Analytical Manual. US Food and Drug Administration (FDA). Available at http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm. Accessed 08 May 2014.

Figueira, E., Lima, A., Branco, D., Quintino, V., Rodrigues, A.M., Freitas, R. 2011. Health concerns of consuming cockles (*Cerastoderma edule* L.) from a low contaminated coastal system. *Environment International,* 37(5), 965–972.

Frederich, M., Pörtner, H.O. 2000. Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in the spider crab *Maja squinado*. *American Journal of Physiology,* 279, R1531–R1538.

Gerschman, R., Gilbert, D.L., Nye, S.W., Dwyer, P., Fenn, W.O. 1954. Oxygen poisoning and X-irradiation: a mechanism in common. *Science,* 119(3097), 623–626.

GISP (Global Invasive Species Programme). 2008. *Marine biofouling: an assessment of risks and management initiatives.* Compiled by Lynn Jackson on behalf of the Global Invasive Species Programme and the UNEP Regional Seas Programme, 68 pp.

Glibert, P.M., Anderson, D.M., Gentien, P., Granéli, E., Sellner, K.G. 2005. The global, complex phenomena of harmful algal blooms. *Oceanography,* 18, 136–147.

Gooch, J.A., DePaola, A., Bowers, J., Marshall, D.L. 2002. Growth and survival of *Vibrio parahaemolyticus* in post-harvest American oysters. *Journal of Food Protection,* 65, 970–974.

Gould, K.S. 2003. Free radicals, oxidative stress and antioxidants. *Abiotic Stresses/Free Radicals, Oxidative Stress and Antioxidants,* 9–15.

Grieshaber, M.K., Hardewig, I., Kreutzer, U., Pörtner, H.O. 1994. Physiological and metabolic responses to hypoxia in invertebrates. *Reviews of Physiology, Biochemistry and Pharmacology,* 125, 44–147.

Guppy, M., Withers, P. 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biological Reviews,* 74, 1–40.

Gutteridge, J.M.C., Halliwell, B. 1990. The measurement and mechanism of lipid peroxidation in biological systems. *Trends in Biochemical Science,* 15, 129–135.

Halliwell, B., Gutteridge, J.M.C. 2007. *Free Radicals in Biology and Medicine*. Oxford University Press, New York, USA.

Heinitz, M.L., Ruble, R.D., Wagner, D.E., Tatini, S.R. 2000. Incidence of *Salmonella* in fish and seafood. *Journal of Food Protection,* 63, 579–592.

Heise, K., Puntarulo, S., Nikinmaa, M., Abele, D., Pörtner, H.O. 2006. Oxidative stress during stressful heat exposure and recovery in the North Sea eelpout *Zoarces viviparus* L. *Journal of Experimental Biology,* 209, 353–363.

Hellmann, J.J., Byers, J.E., Bierwagen, B.G., Dukes, J.S. 2008. Five potential consequences of climate change for invasive species. *Conservation Biology,* 22(3), 534–543.

Helmuth, B., Mieszkowska, N., Moore, P., Hawkins, S.J. 2006. Living on the edge of two changing worlds: forecasting the responses of rocky intertidal ecosystems to climate change. *Annual Review of Ecology, Evolution and Systematics,* 37(1), 373–404.

Hernroth, B.E., Conden-Hansson, A.-C., Rehnstam-Holm, A.-S., Girones, R., Allard, A.K. 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the First Scandinavian Report. *Applied and Environmental Microbiology,* 68(9), 4523–4533.

Hervio-Heath, D., Colwell, R.R., Derrien, A., Robert-Pillot, A., Fournier, J.M., Pommepuy, M. 2002. Occurrence of pathogenic vibrios in coastal areas of France. *Journal of Applied Microbiology,* 92, 1123–1135.

HMSO. 1994. *Nutritional aspects of cardiovascular disease: report on health and social subjects.* Committee of Medical Aspects of Food Policy, 46, Department of Health, HMSO, London, UK.

Hoegh-Guldberg, O., Mumby, P.J., Hooten, A.J., Steneck, R.S., Greenfield, P., Gomez, E., Harvell, C.D., Sale, P.F., Edwards, A.J., Caldeira, K., Knowlton, N., Eakin, C.M., Iglesias-Prieto, R., Muthiga, N., Bradbury, R.H., Dubi, A., Hatziolos, M.E. 2007. Coral reefs under rapid climate change and ocean acidification. *Science,* 318, 1737–1742.

Hofmann, G.E., Somero, G.N. 1996. Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis:* evidence from the heat-shock response and protein ubiquitination. *Marine Biology,* 126, 65–75.

Høi, L., Larsen, J.L., Dalsgaard, I., Dalsgaard, A. 1998. Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Applied and Environmental Microbiology,* 64(1), 7–13.

Hummel, H., de Wolf, L., Zurburg, W., Apon, L., Bogaards, R.H., van Ruttenburg, M. 1989. The glycogen content in stressed marine bivalves: the initial absence of a decrease. *Comparative Biochemistry and Physiology,* 94B, 729–733.

Huss, H.H., Ababouch. L., Gram, L. 2003. *Assessment and management of seafood safety and quality.* Fisheries Technical Paper 444, Food Agriculture Organisation of the United States (FAO), Rome, Italy.

INE/DGPA (Instituto Nacional de Estatística/Direcção-Geral das Pescas e Aquicultura). 2010. *Estatísticas da Pesca.* Available at http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_publicacoes&PUBLICACOESpub_boui=120566488&PUBLICACOESmodo=2&xlang=pt. Accessed 12 February 2014.

IPCC. 2013. *Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC),* Stocker, T.F., Qin, D., Plattner, G.K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp.

ISO. 2005. ISO/TS 16649-3:2005. *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of β-glucuronidase-positive Escherichia coli – Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.* International Organization for Standardization, Geneva.

Jackson, K.L., Ogburn, D.M. 1999. *Review of depuration and its role in shellfish quality assurance.* Final report to Fisheries Research and Development Corporation, Project Number 96/355, NSW Fisheries Final Report Series No. 13, 95 pp.

Jones, S.H., Howell, T.L., O'Neill, K.R. 1991. Differential elimination of indicator bacteria and pathogenic *Vibrio* spp. from eastern oysters (*Crassostrea virginica* Gmelin, 1971) in a commercial controlled purification facility in Maine. *Journal of Shellfish Research,* 10(1), 105–112.

Joyner-Matos, J., Andrzejewski, J., Briggs, L. 2009. Assessment of cellular and functional biomarkers in bivalves exposed to ecologically relevant abiotic stressors. *Journal of Aquatic Animal Health,* 21, 104–116.

Kappus, H. 1987. Oxidative stress in chemical toxicity. *Archives of Toxicology,* 60, 144–149.

Karouna-Renier, N.K., Snyder, R.A., Allison, J.G., Wagner, M.G., Ranga Rao, K. 2007. Accumulation of organic and inorganic contaminants in shellfish collected in estuarine waters near Pensacola, Florida: contamination profiles and risks to human consumers. *Environmental Pollution,* 145, 474–488.

Kershaw, S., Campos, C.J.A., Reese, A., Mitchard, N., Kay, D., Wyer, M. 2013. *Impact of chronic microbial pollution on shellfish.* Cefas, 88 pp.

Key, D. 1981. *Environmental study of Tejo estuary - Consultation on the causes of decline of the oyster fishery.*

Kirchin, M.A., Moore, M.N., Dean, R.T., Winston, G.W. 1992. The role of oxyradicals in intracellular proteolysis and toxicity in mussels. *Marine Environmental Research,* 34, 315–320.

Klontz, K.C., Tauxe, R.V., Cook, W.L., Riley, W.H., Wachsmuth, I.K. 1987. Cholera after the consumption of raw oysters: a case report. *Annals of Internal Medicine,* 107, 846–848.

Kuballa, T., Leonhardt, E., Schoeberl, K., Lachenmeier, D.W. 2009. Determination of methylmercury in fish and seafood using optimized digestion and derivatization followed by gas chromatography with atomic emission detection. *European Food Research and Technology,* 228, 425–431.

Lai, V. W.-M, Cullen, W. R., Ray, S. 1999. Arsenic speciation in scallops. *Marine Chemistry,* 66(1), 81–89.

Le Guyader, F.S., Atmar, R.L. 2007. Viruses in shellfish. *In:* Bosch, A. (Ed.), *Human viruses in water. Perspectives in Medical Virology*, Elsevier, Vol. 17, The Netherlands, Amsterdam, pp. 205–226.

Le Guyader, F.S., Parnaudeau, S., Schaeffer, J., Bosch, A., Loisy, F., Pommepuy, M., Atmar, R.L. 2009. Detection and quantification of noroviruses in shellfish. *Applied and Environmental Microbiology,* 75, 618–624.

Lee, R., Lovatelli, A., Ababouch, L. 2008a. *Bivalve depuration: fundamental and practical aspects.* FAO Fisheries Technical Paper, No. 511.FAO, Rome, 161 pp.

Lee, R.J., Rangdale, R.E., Croci L., Hervio-Heath, D., Lozach, S. 2008b. Bacterial Pathogens in Seafood. *In:* Børresen, T. (Ed.), *Improving seafood products for the consumer.* Woodhead Publishing Series in Food Science, Technology and Nutrition No. 158, 70 pp.

Lee, R.J., Younger, A.D. 2002. Developing microbiological risk assessment for shellfish purification. *International Biodeterioration and Biodegradation,* 50, 177–183.

Lees, D. 2000. Viruses and bivalve shellfish. *International Journal of Food Microbiology,* 59(1–2), 81–116.

Lees, D., Younger, A., Dore, B. 2010. Depuration and relaying. *In:* Rees, G., Pond, K., Kay, D., Bartram, J., Santo Domingo, J.S. (Eds.), *Safe management of shellfish and harvest waters.* World Health Organization (WHO), IWA Publishing, London, UK, pp. 145–181.

Lesser, M.P. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. *Annual Reviews in Physiology,* 68, 253–278.

Liberato, C., Di Cristo, C., De Luise, G., Di Cosmo, A., Paolucci, M. 2003. Preliminary evidence of an oxidative stress system in freshwater crayfish *Austropotamobius pallipes italicus*. *Bulletin Français de la Peche et de la Pisciculture,* 370–371, 151–156.

Lipp, E.K., Hau, A., Colwell, R.R. 2002. Effects of global climate on infectious disease: the cholera model. *Clinical Microbiology Reviews,* 15, 757–770.

Lockwood, B.L., Somero, G.N. 2011. Invasive and native blue mussels (genus *Mytilus*) on the California coast: the role of physiology in a biological invasion. *Journal of Experimental Marine Biology and Ecology,* 400, 167–174.

Lopman, B., Armstrong, B., Atchison, C., Gray, J.J. 2009. Host, weather and virological factors drive norovirus epidemiology: time-series analysis of laboratory surveillance data in England and Wales. *PLos One,* 4(8), e6671.

Lourenço, H., Lima, C., Oliveira, A., Gonçalves, S., Afonso, C., Martins, M.F., Nunes, M.L. 2006. Concentrations of mercury, lead and cadmium in bivalves from the Portuguese coast. *In:* Luten, J.B., Jacobsen, C., Bekaert, K., Saebø, A., Oehlenschläger, J. (Eds.), *Seafood research from fish to dish. Quality, safety and processing of wild and farmed fish.* Wageningen Academic Publishers, The Netherlands, pp. 497–502.

Loutfy, N., Fuerhacker, M., Tundo, P., Raccanelli, S., Ahmed, M.T. 2007. Monitoring of polychlorinated dibenzo-p-dioxins and dibenzofurans, dioxin-like PCBs and polycyclic aromatic hydrocarbons in food and feed samples from Ismailia city, Egypt. *Chemosphere,* 66(10), 1962–1970.

Lurman, G.J., Walter, J., Hoppeler, H. 2014. Seasonal changes in the behaviour and respiration physiology of the freshwater duck mussel *Anodonta anatina*. *Journal of Experimental Biology,* 217, 235–243.

Maazouzi, C., Piscart, C., Legier, F., Hervant, F. 2011. Ecophysiological responses to temperature of the "killer shrimp" *Dikerogammarus villosus*: is the invader really stronger than the native *Gammarus pulex*?. *Comparative Biochemistry and Physiology, Part A: Molecular & Integrative Physiology,*159, 268–274.

Mack, R.N., Simberloff, C.D., Lonsdale, M.W., Evans, H., Clout, M., Bazzaz, F. 2000. Biotic invasions: causes, epidemiology, global consequences and control. *Issues in Ecology,* 5, 1–20.

Maffei, M., Vernocchi, P., Lanciotti, R., Guerzoni, M.E., Belletti, N., Gardini, F. 2009. Depuration of striped venus clam (*Chamelea gallina* L.): effects on microorganisms, sand content, and mortality. *Journal of Food Science,* 74(1), M1–M7.

Maguire, J.A., Cashmore, D., Burnell, G.M. 1999a. The effect of transportation on the juvenile scallop *Pecten maximus* (L.). *Aquaculture Research,* 30, 325–333.

Maguire, J.A., Fleury, P.G., Burnell, G.M. 1999b. Some methods for quantifying quality in the scallop *Pecten maximus* (L.). *Journal of Shellfish Research,* 18, 59–66.

Manfra, L., Accornero, A. 2005. Trace metal concentrations in costal marine waters of the center Mediterranean. *Marine Pollution Bulletin,* 50, 686–962.

Mann, R. 1978. A comparison of morphometric, biochemical and physiological indexes of condition in marine bivalve molluscs. *In:* Thorpe, J.H., Gibbons, J.W. (Eds.), *Energy and Environmental Stress in Aquatic System.* Technical Information Center, U.S. Department of Energy, Oak Ridge, TN, pp. 484–497.

Martinez-Manzanares, E., Egea, F., Castro, D., Moriñigo, M.A., Romero, P., Borrego, J.J. 1991. Accumulation and depuration of pathogenic and indicator microorganisms by the bivalve mollusc, *Chamelea gallina* L., under controlled laboratory conditions. *Journal of Food Protection,* 54(8), 612–618.

Martinez-Urtaza, J., Simental, L., Velasco, D., DePaola, A., Ishibashi, M., Nakaguchi, Y., Nishibuchi, M., Carrera-Flores, D., Rey-Alvarez, C., Pousa, A. 2005. Pandemic *Vibrio parahaemolyticus* O3:K6, Europe. *Emerging Infectious Disease,* 11, 1319–1320.

Mathieu, M., Lubet, P. 1993. Storage tissue metabolism and reproduction in marine bivalves: a brief review. *Invertebrate Reproduction & Development,* 23, 123–129.

Matias, D., Joaquim, S., Matias, A.M., Moura, P., Sousa, J.T., Sobral, P., Leitão, A. 2013. The reproductive cycle of the European clam *Ruditapes decussatus* (L., 1758) in two Portuguese populations: implications for management and aquaculture programs. *Aquaculture,* 406–407, 52–61.

Matoo, O.B., Ivanina, A.V., Ullstad, C., Beniash, E., Sokolova. I.M. 2013. Interactive effects of elevated temperature and CO_2 levels on metabolism and oxidative stress in two common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology,* 164(4), 545–553.

McLaughlin, J.B., DePaola, A., Bopp, C.A., Martinek, K.A., Napol, N.P. 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. *The New England Journal of Medicine,* 353, 1463–1470.

Melzner, F., Mark, F.C., Pörtner, H. 2007. Role of blood oxygen transport in thermal tolerance of the cuttlefish, *Sepia officinalis*. *Integrative and Comparative Biology,* 47(4), 645–655.

Miller, N.A., Stillman, J.H. 2012. Physiological optima and critical limits. *Nature Education Knowledge,* 3(10), 1.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science,* 7, 405–410.

Møller, P., Wallin, H. 1998. Adduct formation, mutagenesis and nucleotide excision repair of DNA damage produced by reactive oxygen species and lipid peroxidation product. *Mutation Research,* 410, 271–290.

Molnar, J.L., Gamboa, R.L., Revenga, C., Spalding, M.D. 2008. Assessing the global threat of invasive species to marine biodiversity. *Frontiers in Ecology and Environment,* 6(9), 485–492.

Mooney, H.A., Cleland, E.E. 2001. The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences of the United States of America,* 98, 5446–5451.

Moore, C.J. 2000. A review of mercury in the environment (its occurrence in marine fish). *Office of Environmental Management Marine Resources Division South Carolina Department of Natural Resources,* 22 pp.

Mora, C., Ospína, A. 2001. Tolerance to high temperatures and potential impact of sea warming on reef fishes of Gorgona Island (tropical eastern Pacific). *Marine Biology,* 139, 765–769.

Morimoto, R.I., Milarski, K.L. 1990. Expression and function of vertebrate hsp70 genes. *In:* Morimoto, R.I., Tissieres, A., Georgopoulos, C. (Eds.), *Stress proteins in biology and medicine.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 323–359.

Morimoto, R.I., Santoro, M.G. 1998. Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nature Biotechnology,* 16(9), 833–838.

Newsholme, E.A., Crabtree, B. 1986. Maximum catalytic activity of some key enzymes in provision of physiologically useful information about energy fluxes. *Journal of Experimental Zoology,* 239, 159–167.

Normanno, G., Parisi, A., Addante, N., Quaglia, N.C., Dambrosio, A., Montagna, C., Chiocco, D. 2006. *Vibrio parahaemolyticus*, *Vibrio vulnificus* and microorganisms of fecal origin in mussels (*Mytilus galloprovincialis*) sold in the Puglia region (Italy). *International Journal of Food Microbiology,* 106(2), 219–222.

Okumus, I., Stirling, H.P. 1998. Seasonal variations in the meat weight, condition index and biochemical composition of mussels (*Mytilus edulis* L.) in suspended culture in two Scottish Sea lochs. *Aquaculture,* 159(3/4), 249–261.

Oliveira, J., Cunha, A., Castilho, F., Romalde, J.L., Pereira, M.J. 2011. Microbial contamination and purification of bivalve shellfish: crucial aspects in monitoring and future perspective – A mini-review. *Food Control,* 22, 805–816.

Oliver, J.D., Kaper, J.B. 1997. *Vibrio* species. *In:* Doyle, M.P., Beuchat L.R., Montville, T.J. (Eds.), *Food Microbiology. Fundamentals and Frontiers.* ASM Press, Washington DC, USA, pp. 228–264.

Olsen, S.J., MacKinon, L.C., Goulding, J.S., Bean, N.H., Slutsker, L. 2000. Surveillance for foodborne disease outbreaks - United States, 1993–1997. *MMWR CDC Surveillance Summaries,* 49(1), 1–62.

Orban, E., Di Lena, G., Nevigato, T., Casini, I., Marzetti, A., Caproni, R. 2002. Seasonal changes in meat content, condition index and chemical composition of mussels (*Mytilus galloprovincialis*) cultured in two different Italian sites. *Food Chemistry,* 77(1), 57–65.

Ortmann, C., Grieshaber, M.K. 2003. Energy metabolism and valve closure behaviour in the Asian clam *Corbicula fluminea*. *Journal of Experimental Biology,* 206, 4167–4178.

Otero, M., Cebrian, E., Francour, P., Galil, B., Savini, D. 2013. Monitoring marine invasive species in Mediterranean Marine Protected Areas (MPAs): a strategy and practical guide for managers. IUCN, Malaga, Spain, 138 pp.

Pannunzio, T.M., Storey, K.B. 1998. Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. *Journal of Experimental Marine Biology and Ecology,* 221, 277–292.

Patterson, M.A., Parker, B.C., Neves, R.J. 1997. Effects of quarantine times on glycogen levels of native freshwater mussels (Bivalvia: Unionidae) previously infested with zebra mussels. *American Malacological Bulletin,* 14, 75–79.

Pawiro, S. 2010. Bivalves: global production and trade trends. *In:* Rees, G., Pond, K., Kay, D., Bartram, J., Domingo, J.S. (Eds.), *Safe management of shellfish and harvest waters.* World Health Organization (WHO), IWA Publishing, London, UK, pp. 11–19.

Picado, A., Le Gal, Y. 1999. Adenylate energy charge as a tool for evaluating sub-lethal toxicity. *Qatar University Science Journal,* 19, 36–45.

Pinto, R.M., Costafreda, M.I., Bosh, A. 2009. Risk assessment in shellfish-borne outbreaks of hepatitis A. *Applied and Environmental Microbiology,* 75, 7350–7355.

Pörtner, H.O. 2010. Oxygen and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *Journal of Experimental Biology,* 213, 881–893.

Pörtner, H.O., Bennett, A.F., Bozinovic, F., Clarke, A., Lardies, M.A., Lucassen, M., Pelster, B., Schiemer, F., Stillman, J.H. 2006. Trade-offs in thermal adaptation: the need for a molecular to ecological integration. *Physiological and Biochemical Zoology,* 79, 295–313.

Pörtner, H.O., Knust, R. 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science,* 315, 95–97.

Pörtner, H.O., Mark, F.C., Bock, C. 2004. Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques. *Respirometry Physiology & Neurobiology,* 141, 243–260

Potasman, I., Paz, A., Odeh, M. 2002. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clinical Infectious Diseases,* 35, 921–928.

Pruzzo, C., Gallo, G., Canesi, L. 2005. Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components. *Environmental Microbiology,* 7(6), 761–772.

Radford, I.J., Cousens, R.D. 2000. Invasiveness and comparative life-history traits of exotic and indigenous Senecio species in Australia. *Oecologia,* 125, 531–542.

Raffin, J.P., Thebault, M.T. 1996. Modelization of coordinated changes of adenylate energy charge and ATP/ADP ratio: application to energy metabolism in invertebrate and vertebrate skeletal muscle. *Comptes Rendus De L'Academie Des Sciences Serie III Sciences De La Vie,* 319(1), 9–15.

Rato, M.P.G.C. 2009. *Estudo da evolução espacial e temporal da poluição por TBT na costa portuguesa.* Tese de doutoramento, Universidade de Aveiro, 229 pp.

Reilly, A., Käferstein, F. 1997. Food safety hazards and the application of the principles of hazard analysis and critical control point (HACCP) system for their control in aquaculture production. *Aquaculture Research,* 28, 735–752.

Rejmanek, M. 1996. A theory of seed plant invasiveness: the first sketch. *Biological Conservation,* 78, 171–181.

Renault, T. 2011. Effects of pesticides on marine bivalves: what do we know and what do we need to know?. *In:* Stoytcheva, M. (Ed.), *Pesticides in the Modern World - Risks and Benefits.* InTech, Rijeka, Croatia, pp. 227–240.

Reyes, L.H., Rahman, G.M.M., Fahrenholz, T., Kingston, H.M.S. 2008. Comparison of methods with respect to efficiencies, recoveries and quantitation of mercury species interconversions in food demonstrated using tuna fish. *Analytical and Bioanalytical Chemistry,* 390, 2123–2132.

Rhodes, M.W., Kator, H.I. 1998 Survival of *Escherichia coli* and *Salmonella* spp. in estuarine environments. *Applied and Environmental Microbiology,* 54, 2902–2907.

Richards, G.P. 1988. Microbial purification of shellfish: a review of depuration and relaying. *Journal of Food Protection,* 51(3), 218–251.

Ripabelli, G., Sammarco, M.L., Grasso, G.M., Fanelli, I., Caprioli, A., Luzzi, I. 1999. Occurrence of *Vibrio* and other pathogenic bacteria in *Mytilus galloprovincialis* (mussels) harvested from Adriatic Sea, Italy. *International Journal of Food Microbiology,* 49(1–2), 43–48.

Rivadeneira, M.M., Fernández, M. 2005. Shifts in southern endpoints of distribution in rocky intertidal species along the south-eastern Pacific coast. *Journal of Biogeography,* 32, 203–209.

Robert, R., Trut, G., Laborde, J.L. 1993. Growth, reproduction and gross biochemical composition of the Manila clam, *Ruditapes philippinarum*, in the Bay of Arcachon, France. *Marine Biology,* 116, 291–299.

Robertson, L.J. 2007. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: a review. *International Journal of Food Microbiology,* 120(3), 201–216.

Roque, A., Lopez-Joven, C., Lacuesta, B., Elandaloussi, L., Wagley, S., Furones, M.D., Ruiz-Zarzuela, I., de Blas, I., Rangdale, R., Gomez-Gil, B. 2009. Detection and identification of tdh- and trh-positive *Vibrio parahaemolyticus* strains from four species of

cultured bivalve molluscs on the Spanish Mediterranean Coast. *Applied and Environmental Microbiology,* 75, 7574–7577.

Rosa, R., Pimentel, M.S., Boavida-Portugal, J., Teixeira, T., Trübenbach, K., Diniz, M.S. 2012. Ocean warming enhances malformations, premature hatching, metabolic suppression and oxidative stress in the early life stages of a keystone squid. *PLOS One,* 7(6), e38282.

Rosa, R., Seibel, B.A. 2008. Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. *Proceedings of the National Academy of Sciences of the United States of America,* 105, 20776–20780.

Rosa, R., Trübenbach, K., Pimentel, M.S., Boavida-Portugal, J., Faleiro, F., Baptista, M., Dionísio, G., Calado, R., Pörtner, H.O., Repolho, T. 2014 Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). *Journal of Experimental Biology,* 217, 518–525.

Rosa, R., Trübenbach, K., Repolho, T., Pimentel, M., Faleiro, F., Boavida-Portugal, J., Baptista, M., Lopes, V.M., Dionísio, G., Leal, M.C., Calado, R., Pörtner, H.O. 2013. Lower hypoxia thresholds of cuttlefish early life stages living in a warm acidified ocean. *Proceedings of the Royal Society B,* 280, 20131695.

Ruano, F. 1997. Fisheries and farming of important marine bivalves in Portugal. *Marine Fisheries Review, NOAA Technical Repport NMFS,* 129, 191–200.

Saito, T., Arai, K., Matsuyoshi, M. 1959. A new method for estimating the freshness of fish. *Bulletin of the Japanese Society for the Science of Fish,* 24, 749–750.

Sakai, A.K., Allendorf, F.W., Holt, J.S., Lodge, D.M., Molofsky, J., With, K.A., Baughman, S., Cabin, R.J., Cohen, J.E., Ellstrand, N.C., McCauley, D.E., O'Neil, P., Parker, I.M., Thompson, J.N., Weller, S.G. 2001. The population biology of invasive species. *Annual Review of Ecology and Systematics,* 32, 305–332.

SARF (Scottish Aquaculture Research Forum). 2010. *Review and cost-benefit analysis for industry of reduced depuration times for the mussel Mytilus edulis.* Aquaculture Research Forum, Scottish, 97 pp.

Sargent, J., Bell, G., McEvoy, L., Tocher, D., Estevez, A. 1999. Recent developments in the essential fatty acid nutrition of fish. *Aquaculture,* 177, 191–199.

Schneider, K.R. 2008. Heat stress in the intertidal: comparing survival and growth of an invasive and native mussel under a variety of thermal conditions. *Biological Bulletin,* 215(3), 253–264.

Sellner, K.G., Doucette, G.J., Kirkpatrick, G.J. 2003. Harmful algal blooms: causes, impacts and detection. *Journal of Industrial Microbiology & Biotechnology,* 30, 383–406.

Shumway, S.E, Rodrick. G.E. 2009. *Shellfish safety and quality.* Woodhead Publishing Limited and CRC Press LLC. FL, Cambridge, UK, 608 pp.

Sies, H. 1991. *Oxidative Stress: Oxidants and Antioxidants.* Academic Press, London, UK.

Silva, H., Batista, I. 2008. *Produção, salubridade e comercialização de moluscos bivalves em Portugal.* Publicações avulsas do IPIMAR, Lisboa, Portugal, vol. 20, 171 pp.

Simberloff, D., Martin, J.-L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M. 2012. Impacts of biological invasions: what's what and the way forward. *Trends in Ecology & Evolution,* 28(1), 58–66.

Simopoulos, A.P. 1991. Omega-3 fatty acids in health and disease and in growth and development. *American Journal of Clinical Nutrition,* 54, 438–463.

Sinski, E. 2003. Environmental contamination with protozoan parasite infective stages: biology and risk assessment. *Acta Microbiologica Polonica,* 52S, 97–107.

Sobral, P., Widdows, J. 1997. Effects of elevated temperatures on the scope for growth and resistance to air exposure of the clam *Ruditapes decussatus* (L.), from southern Portugal. *Scientia Marina,* 61(1), 163–171.

Somero, G.N. 1969. Enzymic mechanisms of temperature compensation: immediate and evolutionary effects of temperature on enzymes of aquatic poikilotherms. *American Naturalist,* 103, 517–530.

Somero, G.N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers.' *Journal of Experimental Biology,* 213, 912–920.

Son, N.T., Fleet, G.H. 1980. Behavior of pathogenic bacteria in the oyster, *Crassostrea commercialis*, during depuration, re-laying, and storage. *Applied and Environmental Microbiology,* 40(6), 994–1002.

Sorte, C.J.B., Jones, S.J., Miller, L.P. 2011. Geographic variation in temperature tolerance as an indicator of potential population responses to climate change. *Journal of Experimental Marine Biology and Ecology,* 400, 209–217.

Sorte, C.J.B., Williams, S.L., Zerebecki, R.A. 2010. Ocean warming increases threat of invasive species in a marine fouling community. *Ecology,* 91(8), 2198–2204.

Souness, R.A., Fleet, G.H. 1979. Depuration of the Sydney Rock oyster, *Crassostrea commercialis*. *Food Technology in Australia,* 31, 397–404.

Stachowicz, J.J., Terwin, J.R., Whitlatch, R.B., Osman, R.W. 2002. Linking climate change and biological invasions: ocean warming facilitates non-indigenous species invasions. *Proceedings of the National Academy of Sciences of the United States of America,* 99(24), 15497–15500.

Stillman, J.H., Somero, G.N. 2000. A comparative analysis of the upper thermal tolerance limits of eastern pacific porcelain crabs, genus *Petrolisthes*: influences of latitude, vertical zonation, acclimation, and phylogeny. *Physiological and Biochemical Zoology,* 73(2), 200–208.

Strahl, J. 2011. *Life strategies in the long-lived bivalve Arctica islandica on a latitudinal climate gradient – Environmental constraints and evolutionary adaptations.* PhD thesis, University of Bremen, 213 pp.

Strand, Ø., Brynjeldsen, E. 2003. On the relationship between low winter temperatures and mortality of juvenile scallops, *Pecten maximus* L., cultured in western Norway. *Aquaculture Research,* 34, 1417–1422.

Su, Y.-C., Liu, C. 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. *Food Microbiology,* 24, 549–558.

Tamayo, D., Ibarrola, I., Navarro, E. 2013. Thermal dependence of clearance and metabolic rates in slow- and fast-growing spats of manila clam *Ruditapes philippinarum*. *Journal of Comparative Physiology B,* 183(7), 893–904.

Telahigue, K., Chetoui, I., Rabeh, I., Romdhane, M.S., Cafsi, M. 2010. Comparative fatty acid profiles in edible parts of wild scallops from the Tunisian coast. *Food Chemistry,* 122(3), 744–746.

Tewksbury, J.J., Huey, R.B., Deutsch, C.A. 2008. Putting the heat on tropical animals. *Science,* 320, 1296–1297.

Timoney, J.F., Abston, A. 1984. Accumulation and elimination of *Escherichia coli* and *Salmonella typhimurium* by hard clams in an in vitro system. *Applied and Environmental Microbiology,* 47(5), 986–988.

Tirado, M.C., Clarke, R., Jaykus, L.A., McQuatters-Gollop, A., Frank, J.M. 2010. Climate change and food safety: A review. *Food Research International,* 43(7), 1745–1765.

Touron, A., Berthe, T., Gargala, G., Fournier, M., Ratajczak, M., Servais, P., Petit, F. 2007. Assessment of faecal contamination and the relationship between pathogens and faecal bacterial indicators in an estuarine environment (Seine, France). *Marine Pollution Bulletin,* 54, 1441–1450.

Uchiyama, M., Mihara, M. 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Analytical Biochemistry*, 86(1), 271–278.

Ulbricht, T.L., Southgate, D.A.T. 1991. Coronary heart disease: seven dietary factors. *Lancet*, 338, 985–992.

Vasseur, D.A., DeLong, J.P., Gilbert, B., Greig, H.S., Harley, C.D.G., McCann, K.S., Savage, V., Tunney, T.D., O'Connor, M.I. 2014. Increased temperature variation poses a greater risk to species than climate warming. *Proceedings of the Royal Society B, Biological Sciences,* 281, 20132612.

Vernocchi, P., Maffei, M., Lanciotti, R., Suzzi, G., Gardini, F. 2007. Characterization of Mediterranean mussels (*Mytilus galloprovincialis*) harvested in Adriatic Sea (Italy). *Food Control,* 18(12), 1575–1583.

Vinu Chandran, R. 2002. *Intracellular osmoregulation in the estuarine mollusc Villorita cyprinoides var. Cochinensis (Mollusca: Bivalvia) Hanley.* PhD thesis, Cochin University of Science and Technology, Cochin, India, 164 pp.

Wagley, S., Rangdale, R. 2007. CEFAS poster presentation*: Identification of the pandemic O3:K6 clone in cases of Vibrio parahaemolyticus related to illness in the UK.* Available at http://www.cefas.co.uk/publications/posters/wagleySGMweb.pdf. Accessed 13 May 2014.

Walker, S.T., Mantle, D., Bythell, J.C., Thomason, J.C. 2000. Oxidative-stress: comparison of species specific and tissue specific effects in the marine bivalves *Mytilus edulis* (L.) and *Dosinia lupinus* (L.). *Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology,* 127(3), 347–355.

Warnes, S.L., Keevil, C.W. 2004. *Work Package 3, Task 3B: Desk studies on feasibility of horizontal standard rapid methods for detection of E. coli (including E. coli O157) and Salmonella.* University of Southampton, 53 pp.

Weitere, M., Vohmann, A., Schulz, N., Linn, C., Dietrich, D., Arndt, H. 2009. Linking environmental warming to the fitness of the invasive clam *Corbicula fluminea*. *Global Change Biology,* 15, 2838–2851.

WHO (World Health Organization). 1999. *PCDD/Fs and their effects on human health.* Fact sheet No.225.

Wilson, I.G., Moore, J.E. 1996. Presence of *Salmonella* spp. and *Campylobacter* spp. in shellfish. *Epidemiology and Infection,* 116, 147–153.

Winston, G.W., Di Giulio, R.T. 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology,* 19, 137–161.

WTTC (World Travel & Tourism Council). 2003. *The Algarve: the impact of travel and tourism on jobs and the economy.*

Yamashita, M., Yabu, T., Ojima, N. 2010. Stress protein HSP70 in fish. *Aqua-BioScience Monographs,* 3(4), 111–141.

Zerebecki, R.A., Sorte, C.J.B. 2011. Temperature tolerance and stress proteins as mechanisms of invasive species success. *PLos One,* 6(4), e14806.

CHAPTER 2

Portuguese consumers' attitudes and perceptions of bivalve molluscs

Anacleto P., Barrento S., Nunes M. L., Rosa R. and Marques A.

(2014)

Food Control, 41: 168–177

DOI: 10.1016/j.foodcont.2014.01.017

Food Control 41 (2014) 168–177

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Portuguese consumers' attitudes and perceptions of bivalve molluscs

Patrícia Anacleto [a,b,*], Sara Barrento [c], Maria Leonor Nunes [a,d], Rui Rosa [b], António Marques [a,d]

[a] Division of Aquaculture and Upgrading (DivAV), Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.), Avenida de Brasília, 1449-006 Lisbon, Portugal
[b] Guia Marine Laboratory, Center of Oceanography, Faculty of Sciences, University of Lisbon (FCUL), Campo Grande, 1749-016 Lisbon, Portugal
[c] Centre for Sustainable Aquaculture Research, Department of Biosciences, College of Science, Swansea University, Singleton Park, Swansea SA2 8PP, UK
[d] Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Rua das Bragas, 289, 4050-123 Porto, Portugal

ARTICLE INFO

Article history:
Received 24 July 2013
Received in revised form
8 January 2014
Accepted 14 January 2014

Keywords:
Consumer survey
Bivalves
Clams
Preferences
Attitudes

ABSTRACT

Bivalves have been promoted as healthy food in many countries. Yet, little information is available about bivalve consumers' purchasing behaviour and attitudes. The aim of this study was to investigate the marketing and quality aspects of several bivalve species with economical value in Portugal and to examine the relationship between each respondent's demographic and socio-economic status with bivalve consumption attitudes and preferences. A randomly selected sample of 1778 people answered a web-based questionnaire. The majority of respondents claimed to consume clams (pullet carpet shell clam and Japanese carpet shell clam) and usually prefer bivalves from national production. Smell, size and cleaned shells were considered as the most important criteria when choosing live bivalves. Most consumers buy clams in supermarkets and revealed good knowledge about the risks associated with its consumption. Trust in the selling establishment and product's label information was the main perceived quality criteria associated to bivalve purchasing. Nonetheless, consumers' attitudes and preferences differed considerably according to their demographic and socio-economic characteristics. The current findings provide a useful tool for producers and stakeholders involved in the trade chain of bivalves to understand the consumption profile and the most important quality criteria involved in bivalve purchase. Additionally, it is an important tool to predict the risks of bivalve consumption as well as to understand the different scenarios of contamination occurring in harvesting areas by national competent authorities.

1. Introduction

Global bivalve production from aquaculture has consistently increased over the years from 7.1 million mt in 1995 to 14.2 million mt in 2010, and the consumer demand is expected to further increase in the future (FAO, 2012). In Portugal, molluscs represent the most important farmed seafood group, representing 48% of total aquaculture production, mainly composed by clams (61%), oysters (25%) and mussels (8%; INE/DGPA, 2010), thus, playing an important role in sustainable seafood production. Portugal has the highest annual seafood per capita consumption in the European Union (54.2 kg/capita in 2009), with bivalves having a special place in consumers preferences (2.5 kg/capita in 2009; INE/DGPA, 2010). The current availability of bivalves in Portugal is insufficient to cope with consumers demand, thus requiring the import of several species (e.g. mussels, oysters). Bivalve prices vary greatly according to season (usually higher in summer), region (higher prices inland), species and consumer demand. Generally, retail prices in early summer (corresponding to a peak of consumption) are: mussels (small: 2.50 €/kg and big: 4.00 €/kg), oysters (4.00 €/kg), Japanese carpet shell clam (7.00 €/kg), pullet carpet shell clam (12.00 €/kg) and grooved carpet shell clam (14.00 €/kg).

Bivalve meat have been recommended in several dietary regimes for their high protein content, low calorific values, low fat/cholesterol profile and lower proportions of saturated fat, the presence of good lipids, significant amounts of omega-3-fatty acids, dietary essential amino acids, vitamin B_{12} and several important minerals such as iron, zinc and copper (Dong, 2001; Krzynowek, Krzynowek, D'Entremont, & Murphy, 1989). Within bivalves, clams are the most important species consumed in Portugal, particularly pullet carpet shell clam (*Venerupis pullastra*) and Japanese carpet shell clam (*Ruditapes philippinarum*), representing 56% of all bivalves (INE/DGPA, 2010). Bivalves species are

* Corresponding author. Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.), Avenida de Brasília, 1449–006 Lisbon, Portugal. Tel.: +351 213 027 025; fax: +351 21 3015948.
E-mail address: panacleto@ipma.pt (A. Marques).

0956-7135/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodcont.2014.01.017

preferably consumed live/raw (e.g. oysters) or lightly cooked (e.g. clams and mussels). This culinary tradition makes consumers more exigent about bivalve quality, but also increases the risk for human health as bivalves can concentrate pathogenic micro-organisms, such as bacteria, human viruses, toxins from harmful algal blooms, and chemical contaminants from the water column since they have a filter-feeding activity (Lee, Lovatelli, & Ababouch, 2008; Rippey, 1994). On the other hand, the harvesting of bivalve molluscs usually occurs in inshore estuaries subjected to strong anthropogenic pressures which are constantly changing due to climatic variability causing impacts such as increase of biological contaminants in the marine environment (e.g., toxins produced by harmful algal blooms (HABs)) and increase of populations of pathogenic microorganisms (e.g., *Vibrio* spp. frequently detected when seawater temperature increases) (Marques, Nunes, Moore, & Strom, 2010). These factors will affect water quality and consequently the quality and risks of consumption of bivalves' species. With a view to protect public health and to provide safe products of high quality to consumers, several regulations have been imposed by the European Commission on shellfish production and trade chain in member states (Lee et al., 2008). These European regulations (EC Regulations 854/2004, EC, 2004; 1441/2007, EC, 2007) establish limits for indicator microorganisms (less than 300 faecal coliforms or less than 230 *Escherichia coli* per 100 g of flesh and inter-valve liquid) and pathogens (absence of *Salmonella* spp. in 25 g of flesh) for a production area of bivalves suitable for direct human consumption (EU class A). However, no limits have been established for other microorganisms such as *Vibrio* spp. (EC, 2001). Other problems were reported in the last years by the Portuguese authorities: several tonnes per month of bivalves unfit for human consumption or harvested in illegal areas to be traded without any sanitary control such as depuration process (Público, 2012). Shellfish-illnesses caused by food contaminated with potentially pathogenic bacteria still occur (4.2% in 2011, RASFF, 2012). Despite these problems, the demand for live bivalves in Portugal is high and, therefore, it is fundamental to comprehend consumers' behaviour to predict the risks of bivalve consumption and adapt the legislation according to the findings.

Consumer surveys are an excellent tool to assess this type of information, since they are a crucial building block in a modern information-based society (Groves et al., 2004). Much research has been devoted to seafood markets and consumption in several European countries, namely fish in Belgium (Verbeke & Vackier, 2005), fish in Denmark (Rortveit & Olsen, 2007), shellfish in Greece (Batzios et al., 2004; Batzios, Angelidis, Moutopoulos, Anastasiadou, & Chrisopolitou, 2003), oysters and mussels (Charles & Paquotte, 1998), seafood in France (Girard, Mariojouls, Paquotte, & Wisner-Bourgeois, 1998), fish in Norway (Rortveit & Olsen, 2009; Trondsen, Braaten, Lund, & Eggen, 2004), seafood in Spain (Manrique & Jensen, 2001) and in Portugal (Cardoso, Lourenço, Costa, Gonçalves, & Nunes, 2013). Several theories and conceptual frameworks can be used to determine consumer's behaviour (e.g. Berg, 2003) The studies focused in shellfish have employed sociological approaches, as they have shown that consumers' behaviour and attitudes vary considerably according to their demographic and socio-economic characteristics. Particularly, French buyers of oysters and mussels have shown different choices according to the location of supermarkets. Yet, there is no such knowledge about bivalve market in Portugal. Multiple studies have indicated no difference in responses between mail-in surveys and web-based surveys (Fleming & Bowden, 2009). In 2012, statistics of Portugal Contemporary Database (PORDATA, 2013) reported that the number of Internet assessment in Portugal was high (62.4% of the whole population), which supports the utilization of web-based surveys to understand consumer behaviour.

Table 1
Demographic characteristics of survey respondents (n = 1778) and Portuguese population with internet access (PORDATA, 2013).

Demographic categories	This study (percent)	Portuguese population (percent)
Age (years)		
<25	20.3	15.3
25–34	26.4	30.3
35–44	21.8	29.1
45–54	19.0	9.2
55–65	11.5	9.6
>65	1.0	6.5
Gender		
Women	62.5	59.7
Men	37.5	40.3
Education level		
Basic	17.0	18.9
High	83.0	81.1
Household people		
1	13.0	n.a.
≥2	87.0	n.a.
Household income		
≤800 €	5.4	n.a.
801–1500 €	17.2	n.a.
1501–3000 €	42.9	n.a.
>3000 €	29.0	n.a.
No answer	5.5	n.a.
Regional distribution		
Coastal	88.9	85.6
Inland	11.1	14.4

n.a., not available.

The present study aim to investigate the consumers' preferences and attitudes towards several bivalve species with economical value in Portugal (e.g. pullet carpet shell clam *V. pullastra*, Japanese carpet shell clam *R. philippinarum*, mussels *Mytilus* sp., oysters *Crassostrea* sp. and scallops *Pecten* sp.), according to consumers demographic and socio-economic conditions. In other words, is bivalve consumers behaviour affected by age, gender, education level, household income and number of household people? Such information is extremely important to enable the assessment of risks associated with the consumption of bivalves by health and maritime authorities and also by industry stakeholders.

2. Materials and methods

An online survey was implemented to investigate the main preferences and quality standards of Portuguese consumers of bivalve shellfish using the software Limesurvey. A short introduction of the study as well as a time frame to complete the study (10 min) was given. With a link provided in the e-mail, the questionnaire could be filled out on-line. The survey was randomly disseminated to more than 5000 people by email, but also disseminated at national press and institutional websites. The survey was made available between April and June 2012. The questionnaire included several general questions covering different aspects, such as bivalve species consumed, frequency of consumption, amount per meal, reasons for not consuming bivalves, and quality criteria for choosing specimens (e.g. origin, shell appearance, package preferences). Special attention was given to clams (e.g. *V. pullastra* and *R. philippinarum)* due to their importance in Portugal such as seasonality, mode of consumption, places to eat, knowledge of risks, consumption of broken or closed shells after cooking, as well as points of purchase, mode of purchase and criteria of choice (smell, shell appearance, size, price, package, expiry date, species origin, presence of depuration certificate and trust in the selling establishment).

A representative survey (n = 1778) was obtained with age, gender, education level, number of household people, social class

Table 2
Consumption of bivalves and reasons (%).

	Total	Age (years)						Gender		Education			Household people (number)		Income (×1000 €)				Region	
		<25	25–34	35–44	45–54	55–65	>65	W	M	Basic	High	1	≥2	≤0.8	0.8–1.5	1.5–3.0	>3.0	Coastal	Inland
Bivalve shellfish (n = 1778)																			
Consumers	79.8	73.7	77.9	84.1	81.8	77.3	76.5	80.8	78.2	79.5	79.9	68.9	80.2	69.1	73.4	80.8	81.9	79.9	78.8
Non-consumers	20.2	26.3	22.1	15.9	18.2	22.7	23.5	19.2	21.8	20.5	20.1	31.1	19.8	30.9	26.6	19.2	18.1	20.1	21.2
Reasons for non-consumption (n = 359)																			
High price	23.0	23.9	27.9	26.5	19.2	11.4	0.0	23.7	22.1	20.3	23.6	23.1	23.0	34.6	26.1	21.0	17.7	24.6	11.1
Unpleasant flavour	29.3	47.8	30.2	28.6	13.5	2.9	33.3	28.5	30.5	33.9	28.3	25.0	30.2	23.1	33.3	37.9	12.7	29.2	30.6
Unpleasant appearance	10.4	19.6	9.3	8.2	1.9	5.7	0.0	11.3	9.2	18.6	8.5	13.5	9.8	11.5	11.6	13.7	5.1	10.7	8.3
Pregnancy or breastfeeding	1.6	0.0	4.7	0.0	1.9	0.0	0.0	2.7	0.0	1.7	1.6	0.0	1.9	3.8	0.0	0.8	3.8	1.4	2.8
Cause allergies	5.0	3.3	4.7	4.1	7.7	8.6	0.0	5.4	4.6	8.5	4.3	11.5	3.8	19.2	5.8	3.2	2.5	4.6	8.3
Previous food poisoning experience	5.7	5.4	5.8	8.2	5.8	2.9	0.0	4.3	7.6	3.4	6.2	3.8	6.0	11.5	1.4	5.6	8.9	5.7	5.6
Fear of food poisoning	24.9	4.3	12.8	34.7	46.2	60.0	66.7	26.3	22.9	25.4	24.8	28.8	24.2	46.2	17.4	25.0	26.6	22.4	44.4
Other	34.4	30.4	39.5	32.7	40.4	40.0	0.0	33.9	38.9	28.8	35.7	34.6	34.3	15.4	33.3	30.6	50.6	34.2	36.1

Notes: Gender (W, Women; M, Men).

and regions (reflecting the distribution of the Portuguese population with access to the internet). Data were split into coastal and inland areas for respondent's place of residence, and also into two groups according to the householder's level of education (basic and higher) and the number of household people (single and households with 2 or more people). In respect to the respondents' age, data were split into six groups: <25; 25–34; 35–44; 45–54; 55–65; and >65 years old. Regarding the monthly household available income level, data were split into four groups according to the Portuguese standards: ≤800 € (low income); 801–1500 € (low-medium income); 1501–3000 € (medium–high income); and >3000 € (high income). Chi-square (χ^2) tests were performed to examine the possible relation between the characteristics of respondent's demographic and socio-economic status with the consumer's attitudes towards bivalves. Level of significance was set for $p < 0.05$. All statistical analyses were carried out using the software STATISTICA™ 7.0 (Statsoft, Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Demographic profile of respondents

Table 1 shows the demographic characteristics of the 1778 respondents of this survey and the Portuguese population with internet access (PORDATA, 2013) and similar percentages were obtained for each category. Most respondents had a university degree (83%) and 48% were adults between 25 and 44 years old. The majority of respondents were constituted by women (63%), which is in agreement with the gender proportion shown in the national 2011 census (59% women; PORDATA, 2013). Regarding the regional distribution, 89% (n = 1580) respondents live in coastal areas, mostly in Lisbon (35%), Setúbal (22%) and Porto (16%). The composition of the household members of respondents was mostly composed by two or more persons (87%). The monthly household available income level revealed that the majority of respondents had an income higher than 1500 € (72%). Despite no data is available for household composition and its available income of Portuguese population with access to internet, the 2011 national census targeting all population also revealed that the household is mainly composed by two or more persons (81%) and 44% households have an available income above 1500 € (PORDATA, 2013).

3.2. Bivalve consumption

The majority of respondents were consumers of bivalves (81% women and 78% men; Table 2). Significantly higher consumption

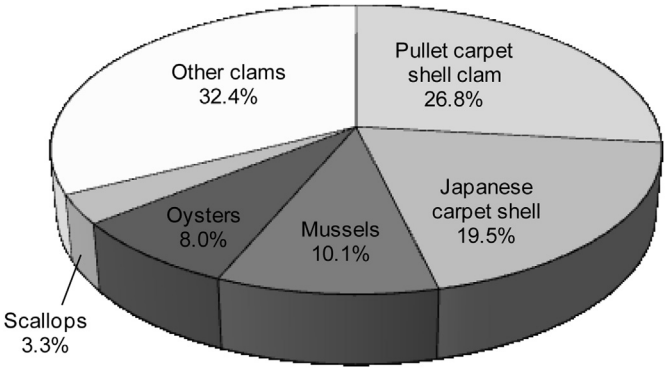


Fig. 1. Preferred bivalve species in Portugal (n = 1419). Other clams included: grooved carpet shell clam (20.1%), hard clam (6.6%), surf clam (2.7%) and peppery furrow shell (1.1%).

Table 3
Consumers' pattern of clams (%) and perceived knowledge of risks associated with its consumption ($n = 1419$).

	Total	Age (years)						Gender		Education		Household people (number)		Income (×1000 €)				Region	
		<25	25–34	35–44	45–54	55–65	>65	W	M	Basic	High	1	≥2	≤0.8	0.8–1.5	1.5–3.0	>3.0	Coastal	Inland
Places to eat																			
Home	79.0	81.5	79.3	78.4	77.8	80.7	45.5	80.0	77.3	87.7	77.2	63.5	80.7	87.5	76.4	81.2	75.4	78.6	82.6
Restaurant	71.1	62.7	69.4	74.5	77.8	73.1	72.7	67.8	77.3	55.9	74.3	80.9	70.1	60.7	62.8	69.1	82.4	71.7	66.7
Other	1.8	1.2	2.6	2.7	1.3	0.0	0.0	2.0	1.4	2.5	1.6	1.7	1.8	1.8	3.7	1.9	0.6	1.9	0.7
Points of purchase																			
Collection	7.3	16.0	7.9	6.3	1.5	5.6	0.0	5.8	10.2	10.9	6.6	8.0	7.3	12.8	9.3	7.3	5.3	7.6	5.1
Directly to the fisherman	10.7	18.9	11.7	9.0	5.3	9.3	0.0	8.0	15.6	13.3	10.1	9.1	10.8	12.8	8.0	10.7	10.9	11.2	6.8
Local market	56.9	54.9	57.3	48.4	60.7	62.0	85.7	55.7	59.2	57.6	56.8	51.1	57.5	53.2	50.0	56.6	62.0	56.1	62.7
Supermarket	80.4	81.1	84.1	80.1	81.6	72.2	42.9	82.3	76.9	80.6	80.4	81.8	80.3	83.0	85.3	82.5	74.3	79.4	88.1
Other places	2.3	1.7	1.3	2.7	3.4	2.8	0.0	2.2	2.4	3.0	2.1	0.0	2.5	0.0	2.7	1.7	3.5	2.5	0.8
Season of consumption																			
Spring	17.8	15.4	19.7	17.8	17.9	17.6	18.2	16.7	19.7	15.2	18.3	16.5	17.9	19.6	13.7	19.2	17.6	17.7	18.1
Summer	53.9	61.5	56.3	51.7	51.3	43.7	27.3	53.9	54.0	50.0	54.7	56.5	53.6	58.9	49.5	55.3	56.0	54.8	47.1
Autumn	4.9	5.8	5.3	4.6	4.3	3.4	9.1	5.4	4.0	4.4	5.0	3.5	5.0	0.0	5.8	5.0	5.0	4.8	5.8
Winter	3.5	3.5	2.3	3.5	2.6	8.4	0.0	3.5	3.3	4.9	3.2	2.6	3.5	3.6	1.1	4.4	3.4	3.5	2.9
Throughout the year	41.3	33.8	39.1	44.0	46.2	46.2	63.6	40.7	42.4	44.1	40.7	40.0	41.4	35.7	46.8	39.9	39.2	40.3	48.6
Mode of consumption																			
Raw	1.9	2.3	1.6	1.2	2.1	3.4	0.0	1.2	3.3	2.5	1.8	3.5	1.8	1.8	1.6	1.7	2.2	1.8	2.9
Slightly cooked	86.2	76.9	87.5	91.5	87.2	88.2	90.9	86.1	86.3	78.9	87.7	90.4	85.7	78.6	83.7	85.8	89.4	86.7	81.9
Extensively cooked	79.4	83.5	80.9	79.2	76.5	73.9	63.6	77.4	82.9	86.8	77.8	72.2	80.1	87.5	81.6	79.3	76.2	79.2	80.4
Knowledge of risks																			
Yes	74.8	63.5	77.6	78.0	76.1	81.5	90.9	74.1	76.1	62.2	77.4	76.5	74.6	67.9	69.0	75.8	77.3	74.6	76.8
No	25.2	36.5	22.4	22.0	23.9	18.5	9.1	25.9	23.9	37.8	22.6	23.5	25.4	32.1	31.0	24.2	22.7	25.4	23.2
Risks																			
Allergies	36.1	36.0	36.0	40.6	30.9	36.1	40.0	37.5	33.6	44.4	34.7	38.6	35.8	39.5	44.3	36.0	33.0	34.2	50.0
Food poisoning	88.0	85.4	88.1	90.6	87.1	88.7	90.0	87.3	89.4	91.3	87.5	83.0	88.6	86.8	85.5	89.1	87.3	88.2	86.8
Toxins	70.8	68.9	69.5	72.8	72.5	74.2	30.0	72.1	68.5	69.0	71.1	72.7	70.6	78.9	74.0	71.3	69.2	70.3	74.5
Heavy metals	52.9	56.1	60.6	57.9	39.9	42.3	50.0	52.3	53.9	40.5	54.9	50.0	53.2	55.3	57.3	54.3	48.6	52.9	52.8
Bacteria or virus	25.8	26.8	26.7	20.3	22.5	38.1	40.0	26.7	24.3	33.3	24.6	26.1	25.8	42.1	29.8	25.4	24.6	26.0	24.5
Other	1.1	1.2	1.3	1.0	0.0	2.1	10.0	0.5	2.2	0.8	1.2	0.0	1.3	2.6	0.0	1.0	1.4	1.3	0.0

Notes: Gender (W, Women; M, Men).

Table 4
Consumers' quality criteria (%) for choosing different bivalve species.

	Pullet carpet shell clam			Japanese carpet shell clam			Mussels			Oysters			Scallops		
	Total ($n = 370$)	Women ($n = 221$)	Men ($n = 149$)	Total ($n = 270$)	Women ($n = 161$)	Men ($n = 109$)	Total ($n = 140$)	Women ($n = 92$)	Men ($n = 48$)	Total ($n = 110$)	Women ($n = 55$)	Men ($n = 55$)	Total ($n = 45$)	Women ($n = 18$)	Men ($n = 27$)
Species origin															
National	78.0	72.1	85.4	45.5	42.3	48.1	67.0	68.7	62.2	70.7	65.2	65.1	36.1	35.0	33.3
Imported	3.9	5.4	2.4	5.5	0.0	11.1	7.3	10.4	2.7	2.4	0.0	4.7	2.8	5.0	0.0
Not important	21.0	26.1	14.6	50.9	57.7	44.4	30.3	26.9	37.8	41.5	34.8	41.9	61.1	60.0	66.7
Quality criteria															
Smell	66.5	61.1	71.3	71.6	68.4	76.9	72.9	71.1	72.1	60.6	64.8	56.4	57.8	53.3	61.5
Shell size	53.5	56.5	51.7	44.8	50.0	42.3	63.6	67.8	62.8	50.5	44.4	56.4	37.8	36.7	38.5
Cleaned shell	41.3	40.5	42.5	31.3	23.7	42.3	42.9	44.4	39.5	39.4	31.5	47.3	31.1	40.0	15.4
Shell colour	21.3	19.1	23.0	11.9	10.5	11.5	9.3	12.2	2.3	16.5	9.3	23.6	15.6	16.7	7.7
Other criteria	3.0	3.1	3.4	6.0	5.3	7.7	5.0	4.4	7.0	21.1	14.8	27.3	8.9	10.0	7.7
No criteria	1.7	2.3	1.1	10.4	7.9	11.5	2.9	2.2	4.7	18.3	16.7	20.0	24.4	23.3	30.8
Shell appearance															
Bright	47.1	54.5	36.8	81.3	66.7	100.0	–	–	–	66.7	80.0	57.1	90.9	100.0	66.7
Dark	56.5	50.0	65.8	18.8	33.3	0.0	–	–	–	33.3	20.0	42.9	18.2	14.3	33.3
Big	88.5	89.0	90.2	83.9	76.2	100.0	88.3	89.9	90.0	66.0	69.2	63.0	88.9	81.8	100.0
Small/medium	12.9	13.4	9.8	19.4	28.6	0.0	14.6	13.0	13.3	34.0	30.8	37.0	22.2	36.4	0.0
Package preferences															
Net package	56.8	55.4	58.6	52.2	55.3	50.0	54.0	52.8	58.1	38.9	37.7	40.0	28.9	26.7	23.1
Wood box	3.9	4.6	3.4	3.0	2.6	3.8	6.5	7.9	4.7	18.5	11.3	25.5	8.9	10.0	7.7
Styrofoam package with modified atmosphere	9.2	11.5	6.9	9.0	7.9	11.5	15.8	19.1	9.3	14.8	13.2	16.4	17.8	23.3	7.7
Plastic box with modified atmosphere	8.3	7.7	8.0	7.5	2.6	15.4	7.9	9.0	4.7	8.3	9.4	7.3	11.1	13.3	7.7
Other packages	2.2	1.5	3.4	4.5	2.6	7.7	4.3	3.4	4.7	4.6	5.7	3.6	8.9	10.0	7.7
No preference	16.2	14.6	17.2	22.4	18.4	23.1	17.3	14.6	23.3	27.8	26.4	29.1	33.3	26.7	53.8

Table 5
Consumer's quality criteria (%) for choosing clams ($n = 1419$).

	Total	Age (years)						Gender		Education		Household people (number)		Income (×1000 €)				Region	
		<25	25–34	35–44	45–54	55–65	>65	W	M	Basic	High	1	≥2	≤0.8	0.8–1.5	1.5–3.0	>3.0	Coastal	Inland
Mode of purchase																			
Live	71.9	61.7	64.9	71.9	80.6	86.1	85.7	68.9	77.5	67.9	72.7	70.5	72.0	63.8	60.7	70.4	81.3	73.0	63.6
Frozen	59.7	66.3	67.4	63.8	50.5	43.5	28.6	63.1	53.5	66.1	58.4	64.8	59.2	72.3	67.3	62.6	50.0	58.4	69.5
Cooked	9.5	4.0	8.8	11.8	9.7	14.8	14.3	6.9	14.4	9.1	9.6	9.1	9.6	8.5	6.7	8.5	13.4	9.9	6.8
Broken or closed shells after cooking																			
Yes	19.2	24.6	17.4	16.2	15.8	23.5	36.4	16.6	23.9	26.5	17.7	12.2	20.0	25.0	23.2	16.3	18.8	19.4	17.4
No	80.8	75.4	82.6	83.8	84.2	76.5	63.6	83.4	76.1	73.5	82.3	87.8	80.0	75.0	76.8	83.7	81.2	80.6	82.6
Quality criteria																			
Smell	39.5	42.9	39.7	38.5	36.4	41.7	42.9	37.7	42.9	38.2	39.8	39.8	39.5	34.0	38.0	39.3	42.6	40.0	36.4
Shell appearance	31.8	29.1	31.0	36.2	34.0	25.9	14.3	31.5	32.4	28.5	32.5	34.1	31.6	27.7	26.7	34.4	32.0	33.1	22.9
Shell size	40.3	49.7	36.0	33.5	42.2	45.4	28.6	37.4	45.6	40.0	40.3	30.7	41.2	44.7	33.3	39.8	44.0	40.8	36.4
Price	54.5	70.9	58.6	52.9	45.6	41.7	14.3	55.7	52.3	53.9	54.6	55.7	54.4	72.3	63.3	56.9	43.7	54.2	56.8
Undamaged packaging	32.5	30.3	28.0	29.9	38.3	40.7	28.6	34.2	29.4	34.5	32.1	36.4	32.1	23.4	34.0	31.5	32.7	32.6	32.2
Expiry date	54.1	51.4	49.4	50.7	59.7	63.9	71.4	57.3	48.0	63.0	52.2	54.5	54.0	53.2	53.3	54.3	54.9	53.3	59.3
Species origin	41.6	47.4	47.3	32.6	38.3	43.5	57.1	42.9	39.3	42.4	41.5	42.0	41.6	42.6	44.0	41.5	40.5	41.6	41.5
Presence of depuration certificate	30.2	17.1	29.3	28.5	37.4	40.7	71.4	29.5	31.5	29.7	30.3	23.9	30.9	34.0	30.0	27.0	34.9	30.4	28.8
Trust in the selling establishment	51.5	53.7	49.8	47.1	56.8	50.0	57.1	53.6	47.4	51.5	51.5	45.5	52.1	51.1	46.7	52.1	53.9	52.0	47.5
No criteria	4.5	5.7	4.6	4.1	2.9	6.5	0.0	4.3	4.8	5.5	4.3	3.4	4.6	2.1	5.3	4.5	4.2	3.9	8.5
Other criteria	4.7	1.1	5.0	5.0	6.8	4.6	14.3	3.9	6.3	1.8	5.3	8.0	4.4	6.4	4.0	3.3	6.3	5.0	2.5

Notes: Gender (W, Women; M, Men).

was detected in respondents 35–54 years old, higher income level (>1500 €) and two or more persons in their household ($p \leq 0.05$). Yet, no major differences occurred in gender, education level or region of residence. Pullet carpet shell clam (26.8%) was the most consumed species, followed by Japanese carpet shell clam (19.5%), which reflects the relatively high production and consumption of clams in Portugal (Fig. 1). Other clams particularly important for consumers (32.4%) were grooved carpet shell clam (20.1%), hard clam (6.6%), surf clam (2.7%) and peppery furrow shell (1.1%). Mussels, oysters and scallops were less important with 10.1%, 8.0% and 3.3%, respectively.

Concerning respondents not eating bivalves, the main reasons were unpleasant flavour (29% in women and 31% in men), fear of food poisoning (26% in women and 23% in men) and high price (24% in women and 22% in men; Table 2). Additionally, unpleasant appearance, previous food poisoning experience, allergies and pregnancy or breastfeeding were also highlighted by 20% respondents as reasons for not eating bivalves. Non-consumers also described other reasons (34%), such as lack of consumption habit (14%) and vegetarianism (3%), particularly respondents with high income level ($p \leq 0.05$). Unpleasant flavour was significantly highlighted by young people (<25 years old) and with income level between 1500 and 3000 €. Additionally, non-bivalve consumers over 65 years old and residing in the countryside significantly indicated that fear of food poisoning was the main reason for not consuming bivalves ($p \leq 0.05$).

3.2.1. Clams consumption

A deeper assessment of clams was performed due to its importance in Portugal. Respondents consume clams at home (79%) or in restaurants (71%; Table 3). Particularly, consumers under 65, with basic education level and two or more people in the household preferably eat bivalves at home ($p \leq 0.05$). In contrast, male consumers, over 35 years old, living alone, and with higher education and income level (>3000 €) significantly prefer restaurants to consume clams ($p \leq 0.05$), because they prefer to have a social life outside home with friends. Clams are mostly purchased in supermarkets (80%), followed by local markets (57%; Table 3). Consumers' age, gender, education and income level ($p \leq 0.05$) significantly influence the choice of clams purchase point. Women under 65, with an income level below 3000 € and residing in inland areas significantly prefer to buy clams in supermarkets ($p \leq 0.05$). In contrast, significant differences were not detected for local markets between all demographic parameters assessed. Batzios et al. (2003) reported the opposite general trend in Greece, revealing a strong preference for buying mussels from traditional fish shops and only a relatively small percentage of consumers choosing supermarkets. A similar trend was observed in Vietnam (Ahn, Luyen, François-Gilles, Alain-Claude, & Dominique, 2009), with a higher preference for buying shellfish at official and temporary markets. Even though convenience is one of the big trends in the food business, time pressure is still not a main driver for convenience in food consumption in Switzerland's supermarkets (Brunner, van der Horst, & Siegrist, 2010). Nonetheless, in Portugal it seems not to be the case, particularly among young consumers, where supermarkets are convenient places to purchase bivalves. A small percentage of Portuguese respondents collect clams or buy them directly to fisherman (18%), mostly without knowing if the harvesting site is safe for consumption. This attitude has statistically higher incidence in young consumers, males, with basic education level and two or more members in the household ($p \leq 0.05$). In contrast, the main source of bivalves consumed in France is self-harvest (63%; Picot, Nguyen, Carpentier, Roudot, & Parent-Massin, 2011).

Table 6
Consumers' attitude to pullet carpet shell clam (*Venerupis pullastra*) with respect to criteria of participants' demographics and socio-economic status ($n = 370$). Frequency of consumption and amount per meal (%).

	Frequency						Amount					
	Weekly	Weekly–monthly	Once/month	Monthly–bi-month	Bimonthly–annually	χ^2-test	<200 g	200–500 g	500–1000 g	1000–1500 g	>1500 g	χ^2-test
Age (years)												
<25	4.0	6.0	12.0	36.0	42.0	17.5 (NS)	12.0	40.0	30.0	10.0	8.0	29.0 (NS)
25–34	1.0	7.1	12.1	22.2	57.6		23.2	43.4	18.2	12.1	3.1	
35–44	2.4	7.2	14.5	30.1	45.8		15.7	44.6	28.9	7.2	3.6	
45–54	3.7	11.0	14.6	36.6	34.1		11.0	31.7	35.4	12.2	9.7	
55–65	3.8	7.5	20.7	34.0	34.0		7.5	49.1	30.2	3.8	9.4	
>65	0.0	0.0	0.0	33.3	66.7		0.0	100.0	0.0	0.0	0.0	
Gender												
Women	2.7	8.6	12.7	27.6	48.4	5.4 (NS)	14.9	43.0	25.8	10.4	5.9	1.4 (NS)
Men	2.7	6.7	16.8	35.6	38.2		14.8	40.3	30.2	8.0	6.7	
Education level												
Basic	6.0	13.4	23.9	26.9	29.8	16.0 (S)	7.5	38.8	26.9	14.9	11.9	10.1 (S)
High	2.0	6.6	12.2	31.7	47.5		16.5	42.6	27.7	8.2	5.0	
Household people												
1	0.0	7.7	17.9	30.8	43.6	1.6 (NS)	30.8	59.0	10.3	0.0	0.0	22.0 (S)
≥2	3.0	7.8	13.9	30.8	44.4		13.0	39.9	29.6	10.6	7.0	
Household income												
≤0.8	0.0	11.1	5.6	27.8	55.5	11.5 (NS)	27.8	50.0	11.1	0.0	11.1	19.3 (NS)
0.8–1.5	8.2	6.1	14.3	26.5	44.9		26.5	44.9	14.3	8.2	6.1	
1.5–3.0	3.1	8.7	15.4	29.0	43.8		13.6	40.1	29.6	11.7	5.0	
>3.0	0.8	7.2	12.0	36.0	44.0		11.2	37.6	34.4	9.6	7.2	
Regional distribution												
Coastal	3.0	8.8	15.1	30.8	42.3	8.8 (NS)	15.1	42.6	26.6	9.4	6.3	1.7 (NS)
Inland	0.0	0.0	7.7	30.8	61.5		12.8	35.9	35.9	10.3	5.1	
Average consumption	2.7	7.9	14.3	30.8	44.3		14.9	41.9	27.6	9.4	6.2	

Notes: Income (× 1000 €); χ^2-test = Likelihood-ratio; χ^2, S = Significant ($p \leq 0.05$), NS = Not significant ($p > 0.05$).

Table 7
Consumers' attitude to Japanese carpet shell clam (*Ruditapes philippinarum*) with respect to criteria of participants' demographics and socio-economic status ($n = 270$). Frequency of consumption and amount per meal (%).

	Frequency						Amount					
	Weekly	Weekly–monthly	Once/month	Monthly–bi-month	Bimonthly–annually	χ^2-test	<200 g	200–500 g	500–1000 g	1000–1500 g	>1500 g	χ^2-test
Age (years)												
<25	0.0	5.9	17.6	47.1	29.4	31.0 (S)	8.8	38.2	26.5	14.7	11.8	24.0 (NS)
25–34	2.5	2.5	11.4	30.4	53.2		20.2	41.8	26.6	8.9	2.5	
35–44	1.4	8.4	9.9	19.7	60.6		16.9	33.8	36.6	5.6	7.1	
45–54	4.2	10.4	20.8	18.8	45.8		6.2	37.5	31.3	12.5	12.5	
55–65	0.0	16.0	16.0	28.0	40.0		12.0	52.0	8.0	20.0	8.0	
>65	0.0	0.0	100.0	0.0	0.0		0.0	100.0	0.0	0.0	0.0	
Gender												
Women	2.0	7.2	11.8	26.1	52.9	2.9 (NS)	18.3	36.0	27.4	10.5	7.8	5.5 (NS)
Men	1.9	7.6	18.1	28.6	43.8		8.6	44.7	29.5	10.5	6.7	
Education level												
Basic	7.0	9.3	23.3	20.9	39.5	11.6 (S)	2.3	44.2	20.9	25.6	7.0	17.7 (S)
High	0.9	7.0	12.6	28.4	51.1		16.8	38.6	29.8	7.4	7.4	
Household people												
1	0.0	0.0	12.0	32.0	56.0	3.2 (NS)	36.0	52.0	12.0	0.0	0.0	17.7 (S)
≥2	2.2	8.2	14.6	26.6	48.5		12.0	38.2	30.0	11.6	8.2	
Household income												
≤0.8	7.1	0.0	28.6	42.9	21.4	16.0 (NS)	21.4	21.4	21.4	21.4	14.4	20.4 (NS)
0.8–1.5	4.7	2.3	11.6	30.2	51.2		11.6	55.8	16.3	7.0	9.3	
1.5–3.0	1.6	9.4	15.0	26.0	48.0		19.7	37.0	29.1	8.7	5.5	
>3.0	0.0	9.0	10.4	23.9	56.7		6.0	34.3	35.8	14.9	9.0	
Regional distribution												
Coastal	2.2	7.5	14.5	26.9	48.9	0.9 (NS)	14.1	41.0	26.9	10.1	7.9	3.4 (NS)
Inland	0.0	6.5	12.9	29.0	51.6		16.1	29.0	38.7	12.9	3.3	
Average consumption	2.0	7.4	14.3	27.1	49.2		14.3	39.5	28.3	10.5	7.4	

Notes: Income (×1000 €); χ^2-test = Likelihood-ratio; χ^2, S = Significant ($p \leq 0.05$), NS = Not significant ($p > 0.05$).

Seasonality also plays an important role in clams' consumption, being summer the preferred season (54%), whereas autumn and winter were the lowest consumption periods (5% and 4%, respectively; Table 3). Interestingly, this attitude is only significantly affected by consumers age ($p \leq 0.05$), whereby older consumers (>65 years old) tend to significantly consume clams all year-round (64%) compared to young consumers (34–46%), which is similar to the findings reported by Batzios et al. (2003) for the Greek population. Additionally, younger consumers statistically consume more clams in summer ($p \leq 0.05$). Regarding clam's cooking style, 86% of consumers slightly cook clams, 79% use long clam cooking practices and only 2% eat them raw. This attitude only varied significantly with consumers' age, gender and education level ($p \leq 0.05$). Particularly, consumers between 35 and 44 years old and with more 65 years old, women and those with high education level prefer to slightly cook clams instead of extensively cook them. Both laboratory and field studies indicate that short cooking practices rarely heats bivalve flesh to temperatures that can effectively inactivate most pathogenic agents (Koff & Sear, 1967). Such practice is a potential source of serious infections and diseases (Gerba, 1988).

The majority of bivalve consumers claimed to have good knowledge about the risks associated with the consumption of clams (75%), with statistical higher knowledge of older people, consumers with higher education level and living alone ($p \leq 0.05$; Table 3). The most relevant risks highlighted by clam consumers were food poisoning (88%), toxins (71%) and heavy metals (53%). The only statistical difference was detected for heavy metals in consumers' education level, where respondents with higher education were more aware about this problem.

3.3. Bivalves quality criteria

Generally, Portuguese consumers prefer bivalves from national production (Table 4), especially pullet carpet shell clam, mussels and oysters, whereas species origin is not important criteria for consumers of scallops and Japanese carpet shell. Apart from vitality signs and shells integrity, the most important bivalve quality criteria for consumers were similar for all species (Table 4), i.e. smell, shell size and cleaned shell were equally important. Concerning oysters, male consumers significantly indicated that shell colour ($p \leq 0.05$) is the most relevant criteria compared to women.

Regarding shell appearance, bright coloured and big shells were preferred, except male consumers of pullet carpet shell clam that prefer darker and big shells though not significantly (Table 4). It is also consensual that thick oysters were preferred (around 60% in both sexes; data not shown). Furthermore, male consumers significantly choose big Japanese carpet shell clams ($p \leq 0.05$) compared to women.

Concerning packaging preferences, the net package was the preferred option (39%) or revealed no preference (28%). Wood box was more attractive for oysters significantly among male consumers (26%; $p \leq 0.05$). A major percentage of scallops' consumers don't have any preference in packaging, particularly males (54%). Interestingly, the most modern and convenient packages of plastic or Styrofoam with modified atmosphere were only mentioned by few consumers, likely since smell is an important quality criterion for bivalves. However, the plastic box is significantly preferred by male consumers of Japanese carpet shell compared to females, though not significantly.

3.3.1. Clams quality criteria

Clams are preferably bought live (72%) or frozen (60%; Table 5). Older consumers (>55 years old), males, people living in coastal areas or with a higher income level (>3000€) significantly purchase live clams ($p \leq 0.05$). On the other hand, frozen clams are statistically preferred by consumers under 44 years old, women, people residing inland and with income below 800 € ($p \leq 0.05$). The vast majority of respondents do not consume clams that are broken and/or closed after cooking (81%). This attitude significantly varied with age, gender and education level ($p \leq 0.05$), whereby these clams are not consumed by respondents between 25 and 54 years, women and with higher education. Clams' prices, expiry date, trust in the selling establishment, origin, size and smell were the most crucial quality parameters for clam consumers. Moreover, the product's label information (species origin, expiry date and depuration certificate) and the trust in the selling establishment (86%) were more important than price (55%). This might indicate that consumers have no experience in buying clams and mainly rely on product information and establishment confidence. This trend is similar to other consumer European markets, and is mainly driven by the fear of shellfish poisoning. In Greece, price is not considered as important, because the most consumed bivalve, mussel, is a cheap and affordable product compared to other seafood (Batzios et al., 2004). Although this is not the situation in Portugal, where pullet carpet shell is more expensive than other popular live seafood (including edible crab), price was significantly more important to young consumers (<25 years old) or respondents with income level below 800 € ($p \leq 0.05$). Expiry date was significantly relevant to older consumers (>65 years old), women, respondents with basic education level and with two or more persons in the household ($p \leq 0.05$). Also, species origin and presence of depuration certificate are quality parameters significantly more important for consumers over 65 years old ($p \leq 0.05$). Furthermore, specimen's intrinsic quality criteria are also relevant to a great percentage of consumers for choosing clams (e.g. smell, size and shell appearance). Size is a criteria significantly more important for younger consumers (<25 years old) and males ($p \leq 0.05$) than older consumers and women. Furthermore, shell appearance is significantly relevant for consumers residing in coastal areas ($p \leq 0.05$) compared to people living in the interior. Significant differences were not detected for smell and undamaged packaging for all demographic parameters assessed. Therefore, stakeholders along the trade chain must be aware that such criteria are crucial for consumers.

3.4. Influence of demographics and socio-economic criteria on the frequency and amount of clam's consumption

Overall, twenty seven per cent respondents mentioned a relatively high clam consumption frequency (more than once per month), with males revealing higher consumption frequency than females (data not shown). A high percentage of respondents reported low frequency (bimonthly to annually, i.e. one to five times per year) of pullet and Japanese carpet shell clam's consumption (44 and 49%, respectively; Tables 6 and 7). Yet, significant higher consumption frequency of pullet carpet shell clam was detected in consumers with basic educational level ($p \leq 0.05$; Table 6). In contrast, significantly higher consumption frequency of Japanese carpet shell clam was observed in consumers with less than 65 years old and with basic educational level ($p \leq 0.05$; Table 7).

Concerning the amount of clams consumed per household, the majority of respondents reported consumption per family meal between 200 and 1000 g in both clam species, varying between 69.5% and 67.8% for pullet and Japanese carpet shell clam, respectively. Significant higher quantities of both clam species were consumed by respondents with basic education level and with two or more household persons ($p \leq 0.05$; Tables 6 and 7).

3. Conclusions

This survey was the first conducted in Portugal addressing bivalve's consumers and how demographic and socio-economic characteristics influence consumers' behaviour. In general, the consumption of bivalves by Portuguese population is well defined. The most important bivalve species consumed in Portugal are pullet carpet shell clam (*V. pullastra*) and Japanese carpet shell clam (*R. philippinarum*. The most important quality criteria for choosing bivalves are smell, size and shell appearance. Concerning clams, most consumers prefer to buy live clams mostly in summer at supermarkets and to cook them at home or consume in restaurants. The main perceived quality criteria associated to clams purchase behaviour are trust in the selling establishment and product's label information. Even though consumers are aware about the risks associated with clams consumption, pointing out food poisoning and toxins as the main issues, some of them harvest specimens from polluted areas or buy them directly to fisherman, and consume raw or lightly steamed clams, even with broken or closed shells. Such practices may pose a substantial risk to human health. Nonetheless, consumers' attitudes and preferences towards different bivalve species differ considerably according to their demographic and socio-economic status. Hence, this survey may help producers and stakeholders involved in the trade chain of bivalves to understand the consumption behaviour as well as the most important quality criteria involved in its purchase. In this way, they will be able to create and adapt their products and the trade chain according to consumers' requirements. Additionally, the methodological approach can be an important tool to predict the risks of bivalve consumption by national competent health authorities.

Acknowledgements

The first author acknowledge a PhD scholarship of the Portuguese Foundation for Science and Technology (FCT) (Ref. SFRH/BD/69274/2010) and the two last authors were supported by a research contract from the Ciência 2008 Program of FCT. The authors also would like to thank the Informatics Department of FCUL and IPMA for their technical assistance with the survey.

References

Ahn, N. T., Luyen, T. T., François-Gilles, C., Alain-Claude, R., & Dominique, P. M. (2009). Survey of shellfish consumption in South coastal Vietnam (Nha Trang). In *Proceedings of the 7th international conference on molluscan shellfish safety. Nantes, France*.

Batzios, C. H., Angelidis, P., Moutopoulos, D. K., Anastasiadou, C. H., & Chrisopolitou, V. (2003). Consumer attitude towards shellfish in the Greek market: a pilot study. *Mediterranean Marine Science, 4*(2), 155–174.

Batzios, C., Angelidis, P., Papanagiotou, E. P., Moutopoulos, D. K., Anastasiadou, C. H., & Chrisopolitou, V. (2004). Greek consumer's image of the cultured mussel market. *Aquaculture International, 12*, 239–257.

Berg, N. (2003). Normative behavioral economics. *Journal of Socio-Economics, 32*, 411–427.

Brunner, T. A., van der Horst, K., & Siegrist, M. (2010). Convenience food products. Drivers for consumption. *Appetite, 55*, 498–506.

Cardoso, C., Lourenço, H., Costa, S., Gonçalves, S., & Nunes, M. L. (2013). Survey into the seafood consumption preferences and patterns in the portuguese population. Gender and regional variability. *Appetite, 64*, 20–31.

Charles, E., & Paquotte, P. (1998). Product differentiation and quality approach in the French market for oysters and mussels. In A. Eide, & T. Vassdal (Eds.), *Proceedings of IXth conference of IIFET* (pp. 35–42) (Tromsø, Norway).

Dong, F. M. (2001). *The nutritional value of shellfish*. Washington Sea Grant Program. University of Washington. 22 March 2013. Available at www.wsg.washington.edu/.

EC. (2001). *Opinion of the scientific committee on veterinary measures relating to public health on Vibrio vulnificus and Vibrio parahaemolyticus (in raw and undercooked seafood)* (pp. 1–64). European Commission Health & Consumer Protection Directorate General. Directorate C—Scientific Opinions.

EC. (2004). Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal of the European Union, L226*, 83–127.

EC. (2007). Commission regulation (EC) 1441/2007 of 5 December 2007 amending regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union, L322*, 1–18.

FAO (Food and Agriculture Organizationof the United Nations). (2012). *The state of world fisheries and aquaculture 2012*. 07 June 2013. Available at www.fao.org/docrep/016/i2727e/i2727e.pdf.

Fleming, C. M., & Bowden, M. (2009). Web-based surveys as an alternative to traditional mail methods. *Journal of Environmental Management, 90*(1), 284–292.

Gerba, C. (1988). Viral disease transmission by sea-foods. *Food Technology, 42*(3), 99–103.

Girard, S., Mariojouls, C., Paquotte, P., & Wisner-Bourgeois, C. (1998). An analysis of seafood consumption survey methods in France. In *Proceedings of IXth conference of IIFET* (pp. 718–736) (Tromsø, Norway).

Groves, R. M., Floyd, J., Fowler, , Jr., Couper, M. P., Lepkowski, J. M., Singer, E., et al. (2004). *Survey methodology*. New York: John Wiley & Sons, Inc.

INE/DGPA (Instituto Nacional de Estatística/Direcção-Geral das Pescas e Aquicultura). (2010). *Estatísticas da Pesca*. 07 June 2013. Available at http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_publicacoes&PUBLICACOESpub_boui=120566488&PUBLICACOESmodo=2&xlang=pt.

Koff, R. S., & Sear, H. S. (1967). Survival of viruses in steamed clams. *New England Journal of Medicine, 276*, 737–740.

Krzynowek, J., D'Entremont, D. L., & Murphy, J. (1989). Proximate composition and fatty acid and cholesterol content of squid, *Loligo pealei* and *Illex illecebrosus*. *Journal of Food Science, 54*(1), 45–48.

Lee, R. J., Lovatelli, A., & Ababouch, L. (2008). *Bivalve depuration: Fundamental and practical aspects*. 07 June 2013. Available at ftp://ftp.fao.org/docrep/fao/011/i0201e/i0201e.pdf.

Manrique, J., & Jensen, H. H. (2001). Spanish household demand for seafood. *Journal of Agricultural Economics, 52*, 23–37.

Marques, A., Nunes, M. L., Moore, S. K., & Strom, M. S. (2010). Climate change and seafood safety: human health implications. *Food Research International, 43*(7), 1766–1779.

Picot, C., Nguyen, T. A., Carpentier, F., Roudot, A., & Parent-Massin, D. (2011). Relevant shellfish consumption data for dietary exposure assessment among high shellfish consumers, Western Brittany, France. *International Journal of Environmental Health Research, 21*(2), 86–105.

PORDATA. (2013). 22 March 2013. Available at http://www.pordata.pt/Portugal.

Público. (2012). *Apanhadores ilegais de amêijoa facturam "milhares" de euros no Tejo (21.11.2011)*. 22 March 2013. Available at http://www.publico.pt/Sociedade/apanhadores-ilegais-de-ameijoa-facturam-milhares-de-euros-no-tejo-1521912.

RASFF. (2012). *The rapid alert system for food and feed (RASFF) – Annual report 2011* (p. 47). Publications Office of the European Union.

Rippey, S. R. (1994). Infectious diseases associated with molluscan shellfish consumption. *Clinical Microbiology Reviews, 7*(4), 419–425.

Rortveit, A. W., & Olsen, S. O. (2007). The role of consideration set size in explaining fish consumption. *Appetite, 49*, 214–222.

Rortveit, A. W., & Olsen, S. O. (2009). Combining the role of convenience and consideration set size in explaining fish consumption in Norway. *Appetite, 52*, 313–317.

Trondsen, T., Braaten, T., Lund, E., & Eggen, A. E. (2004). Health and seafood consumption patterns among women aged 45–69 years. A Norwegian seafood consumption study. *Food Quality and Preference, 15*(4), 117–128.

Verbeke, W., & Vackier, I. (2005). Individual determinants of fish consumption: application of the theory of planned behaviour. *Appetite, 44*, 67–82.

CHAPTER 3

Microbiological composition of native and exotic clams from Tagus estuary: Effect of season and environmental parameters

Anacleto P., Pedro S., Nunes M. L., Rosa R. and Marques A.

(2013)

Marine Pollution Bulletin, 74(1): 116–124
DOI: 10.1016/j.marpolbul.2013.07.019

Marine Pollution Bulletin 74 (2013) 116–124

Contents lists available at SciVerse ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Microbiological composition of native and exotic clams from Tagus estuary: Effect of season and environmental parameters

Patrícia Anacleto [a,b,*], Sónia Pedro [a], Maria Leonor Nunes [a], Rui Rosa [b], António Marques [a]

[a] Division of Aquaculture and Upgrading (DivAV), Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.), Av. Brasília, 1449-006 Lisbon, Portugal
[b] Guia Marine Laboratory, Center of Oceanography, Faculty of Sciences, University of Lisbon (FCUL), Campo Grande, 1749-016 Lisbon, Portugal

ARTICLE INFO

Keywords:
Venerupis pullastra
Ruditapes philippinarum
Escherichia coli
Salmonella spp.
Vibrio spp.
Total viable counts

ABSTRACT

The influence of seasonal and environmental parameters on the occurrence of bacteria was investigated in two clam species (*Venerupis pullastra* and *Ruditapes philippinarum*), water and sediment from the Tagus estuary. Total viable counts (TVC), *Escherichia coli*, *Salmonella* spp. and *Vibrio* spp. were evaluated during one-year. Overall, significant seasonal variations were found in both sampling sites, especially for *E. coli* and *Vibrio* spp. levels. In summer, significantly higher *Vibrio* spp. levels were found in *R. philippinarum* and sediment samples, but not in *V. pullastra* clams and water samples. In contrast, significantly higher TVC and *E. coli* levels were observed in winter months in water and sediment samples. *Salmonella* spp. was generally isolated when higher levels of *E. coli* were detected, particularly in *R. philippinarum*. This study is useful for authorities to develop monitoring strategies for coastal contamination and to estimate human health risks associated with the consumption of bivalves.

1. Introduction

The harvesting bivalve molluscs usually occurs in inshore estuaries with high primary productivity and has been an important component of Southern European fisheries related activities since ancient times (Lees, 2000). In 2010, the European annual production reported for this group accounted for 26.2% of total aquaculture production (FAO, 2012). Bivalve molluscs, like pullet carpet shell clam (*Venerupis pullastra*) and Japanese carpet shell clam (*Ruditapes philippinarum*), are the most important resources commercially exploited in Tagus estuary, playing a crucial socio-economic role to riparian human communities because they are easily collected and have high nutritional value (Oliveira et al., 2011). Since these species have a filter-feeding activity, they can accumulate pathogens from seawater (Cook, 1991).

The number and type of microorganisms present in marine or estuarine waters depend on seasonal, climatic and anthropogenic factors (Vernocchi et al., 2007). The microbiota found in shellfish can be divided into three groups (Reilly and Käferstein, 1997): (i) indigenous bacteria that naturally occur in marine or estuarine environments (e.g., *Vibrio* spp., *Listeria monocytogenes*, *Clostridium botulinum* and *Aeromonas hydrophila*); (ii) non-indigenous/enteric bacteria that occur due to faecal contamination (e.g., *Salmonella* spp., *Escherichia coli*, *Shigella* spp., *Campylobacter* spp. and *Yersinia enterocolitica*); and (iii) bacteria from contamination during food preparation and processing by the distribution industry or consumers (e.g., *Bacillus cereus*, *Stapylococcus aureus* and *Clostridium perfringens*). The routes of transmission from the environment to humans include the consumption of raw, uncooked or lightly cooked shellfish, representing a significant health risk (Lees et al., 2010). In 2011, bivalve molluscs represented 4.2% of all European food contaminated with potentially pathogenic bacteria (RASFF, 2012).

Microbiological pollution in shellfish-growing waters is a common problem in almost all the coastal areas (Almeida and Soares, 2012). In this context, the production, harvesting and commercialization of bivalve molluscs as well as the classification of the overlying waters are regulated by the EC Regulations 854/2004 (EC, 2004) and 1441/2007 (EC, 2007). These regulations establish limits for indicator microorganisms (less than 300 fecal coliforms or less than 230 *E. coli* per 100 g of flesh and intravalvular liquid) and pathogens (absence of *Salmonella* spp. in 25 g of flesh) for a production area suitable for direct human consumption (EU class A). However, *Vibrio* species are excluded from the European applicable microbiologic requirements for shellfish-harvesting areas and are not included in the European Network for Epidemiologic Surveillance and Control of Communicable Diseases and from the Microbiological Surveillance System for Infectious Gastroenteritis (EC, 2001).

The presence and concentration of microorganisms in bivalves vary temporarily and spatially in estuarine regions within the same habitat (Lindstrom, 2001), as well as between habitats (Yannarell and Triplett, 2004), due to environmental factors (Hahn, 2006).

* Corresponding author at: Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.), Av. Brasília, 1449-006 Lisbon, Portugal. Tel.: +351 213 027 025; fax: +351 213 015 948.
E-mail address: panacleto@ipma.pt (P. Anacleto).

0025-326X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.marpolbul.2013.07.019

Several authors assessed the seasonal microbiological structure of bivalve molluscs from moderately to highly polluted waters, such as clams *Egeria radiata* from Great Kwa estuary in Nigeria (Eja et al., 2008) and oysters *Crassostrea madrasensis* from estuaries along the southwest coast of India (Deepanjali et al., 2005). Additionally, many studies addressed the effects of environmental factors like salinity, temperature, turbidity, pH, tides, etc. (e.g., Martinez-Urtaza et al., 2004; Campos and Cachola, 2007) on levels of microbiological contamination in coastal areas used for the commercial production of bivalves. Yet, few studies addressed the seasonal environmental factors influencing the bacteriological contamination of clams. In this context, this study aim to evaluate the influence of environmental parameters, such as temperature, salinity, pH and dissolved oxygen, on bacterial levels (*E. coli*, *Salmonella* spp., *Vibrio* spp. and total viable counts – TVC) found in water, sediment and clam species (i.e., the native *V. pullastra* and the exotic *R. philippinarum*) from Tagus estuary (Portugal).

2. Materials and methods

2.1. Study area

Clams were collected in two active bivalve fishing sites of the Tagus estuary, Trafaria (38°67′682″N, 9°24′362″W) and Barreiro (38°65′442″N, 9°09′365″W) (Fig. 1). This estuary, located in the most populated area of Lisbon, Portugal, is one of the largest on the west coast of Europe. It has a broad shallow bay covering an area of about 320 km^2 (Brogueira and Cabeçadas, 2006). The Tagus river is the main source of freshwater to the estuary, representing the second most important hydrological basin in the Iberian Peninsula. Seawater enters the estuary through a deep narrow inlet channel, and is classified as a mesotidal estuary according to the National Oceanic and Atmospheric Administration (NOAA), with semidiurnal tides ranging from 0.4 m at neap tide to 4.1 m at spring tide. The estuary receives effluents from agricultural, industrial and urban sources (Gameiro and Brotas, 2010).

2.2. Sampling of clams, water and sediment

Studies were carried out between July 2011 and June 2012 in the Tagus estuary. Monthly collections of estuarine water, sediment and clams samples were made. Native pullet carpet shell clam (*V. pullastra*) was harvested from Trafaria, located in the mouth of Tagus estuary, and exotic Japanese carpet shell clam (*R. philippinarum*) was harvested from Barreiro, located in the upstream part of the estuary (Fig. 1). These clam species were selected due to their different filtering capacities, adaptability and tolerance to environmental variability, and also due to their colonization of different niches in the aquatic ecosystem. Sampling of *V. pullastra* was performed during the first 6 months (July–December 2011), due the fact that the Tagus estuary has been affected by a clam overexploitation. All clams and sediment samples were collected through diving and immediately stored in sterile plastic bags and bottles, respectively. Water samples were collected using a sterile Niskin bottle near the bottom, i.e., at 27–30 m in Trafaria and 2–6 m in Barreiro.

A total of 54 samples were collected from both sampling sites and from each sample three replicates were made. Water temperature, salinity, pH and dissolved oxygen were measured *in situ* with a WTW handheld Meter Multi 350i (WTW, Germany). Table 1 shows the means of each parameter in the two sites of the Tagus estuary. All samples were transported to the laboratory in thermally insulated boxes in aseptic conditions and then stored at 4 °C until further analysis. Only live clams were used for analysis and around 1 kg of clams were sampled per month in each site, being thoroughly washed with sterile water and a brush to remove any material adhering to the shells and dried with absorbent paper. Subsequently, clams were opened and the muscle and intervalvar liquid were aseptically extracted into a sterile container using a sterile scalpel.

2.3. Microbiological analyses

2.3.1. E. coli and total viable counts (TVC)

2.3.1.1. Clams and sediment samples. The enumeration of *E. coli* was carried out according to the method of the International Organization for Standardisation (ISO 16649-2; ISO, 2001) and the pour plate method was used for total viable counts (TVC). Edible clam meat with intervalvar fluid or sediment was initially diluted in Maximum Recovery Diluent (MRD; Oxoid Ltd., Basingstoke, Hampshire, UK) and homogenized during 60 s in a Stomacher 400 (Seward Laboratory System, London, UK). For *E. coli* quantification, appropriate serial decimal dilutions were performed in MRD,

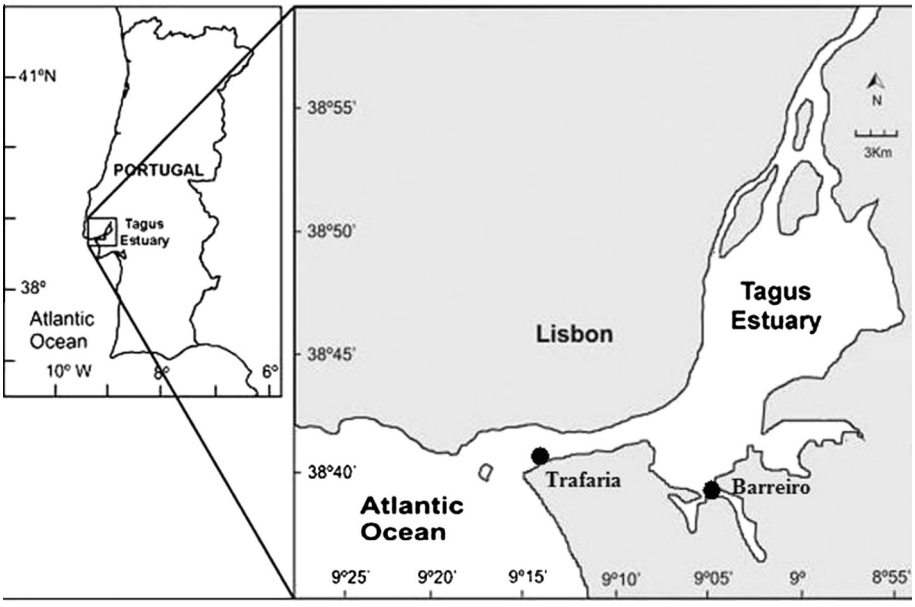


Fig. 1. Map showing Tagus estuary (Portugal) and sampling locations (Trafaria for *Venerupis pullastra* and Barreiro for *Ruditapes philippinarum*).

Table 1
Seasonal variation of physico-chemical parameters of the water samples at th two sampling locations (Trafaria and Barreiro).

		Trafaria				Barreiro			
		T (°C)	Salinity (‰)	D.O. (mg/L)	pH	T (°C)	Salinity (ppt)	D.O. (mg/L)	pH
2011	July	16.0	39	8.79	8.194	21.2	33	10.26	8.115
	August	18.2	36	9.04	8.265	18.6	30	7.60	8.016
	September	17.9	37	7.05	8.165	20.6	33	9.59	7.942
	October	19.3	35	8.27	8.274	20.1	35	5.19	8.069
	November	14.0	35	9.06	8.424	13.3	25	8.74	8.307
	December	12.1	33	9.13	8.306	11.3	32	8.71	8.203
2012	January	n.a.	n.a.	n.a.	n.a.	15.1	37	9.50	8.078
	February	n.a.	n.a.	n.a.	n.a.	12.4	36	9.53	8.274
	March	n.a.	n.a.	n.a.	n.a.	15.2	36	8.01	8.002
	April	n.a.	n.a.	n.a.	n.a.	24.8	38	7.95	8.016
	May	n.a.	n.a.	n.a.	n.a.	22.4	38	7.95	8.225
	June	n.a.	n.a.	n.a.	n.a.	22.7	35	6.07	7.717
	Average ± SD	16.2 ± 2.8	36 ± 2	8.56 ± 0.80	8.271 ± 0.091	18.1 ± 4.5	34 ± 4	8.26 ± 1.48	8.080 ± 0.163
	Range	12.1–19.3	33–39	7.05–9.13	8.165–8.424	11.3–24.8	25–38	5.19–10.26	7.717–8.307

Abbreviations: T, temperature; D.O., dissolved oxygen; n.a., not analyzed.

followed by culture on tryptone bile X-glucuronide agar (Chromocult TBX; Merck, Darmstadt, Germany), followed by an initial incubation step at 37 ± 1 °C during 4.0 ± 0.5 h (for stressed cells) and a second incubation step at 44 ± 1 °C for 18 ± 2 h. TVC was assessed on trypticase soy agar (TSA; Merck, Darmstadt, Germany) and incubated at 22 °C for five days. After incubation, Petri dishes with 30–300 colonies were chosen to count blue colonies typical from β-glucuronidase positive *E. coli*. Microbiological counts were expressed as log colony-forming units per gram of sample (log CFU g^{-1}) for TVC and log CFU 100 g^{-1} for *E. coli*.

2.3.1.2. Water samples. The quantification of TVC was performed using the same procedure for clams and sediment. However, the enumeration of *E. coli* was carried out according to the ISO 9308-1 (ISO 2000) with some modifications. Briefly, appropriate volumes of water sample (10 and 100 mL) were filtered through 0.45 μm-pore size membrane filters (Millipore Corporation, Bedford, MA, USA). The filter disks were aseptically placed on Chromocult TBX and incubated at 44 ± 1 °C for 18 ± 2 h. The identification and enumeration of colonies were identical to the method used for clams and sediment. Microbiological counts were expressed as log colony-forming units per millilitre of sample (log CFU mL^{-1}) for TVC and log CFU 100 mL^{-1} for *E. coli*.

2.3.2. Salmonella spp.

2.3.2.1. Clams and sediment samples. The detection of *Salmonella* spp. was performed according to the ISO 6579 (ISO, 2002), with some modifications. Briefly, 225 mL of buffered peptone water (BPW; Oxoid Ltd., Basingstoke, Hampshire, UK) was added to 25 g of homogenized sample and incubated at 37 °C during 18–24 h. Afterwards, Salmonella Rapid Tests (Oxoid Ltd., Basingstoke, Hampshire, UK) were used to detect presumptive *Salmonella* colonies. In this step, the culture vessel containing a *Salmonella* rapid test medium (SRTEM, Oxoid Ltd., Basingstoke, Hampshire, UK) was inoculated with 1 mL of pre-enriched culture and incubated at 41 °C during 24 h. Each positive broth was subcultured on selective media, such as xylose–lysine–desoxycholate agar (XLD, Oxoid Ltd., Basingstoke, Hampshire, UK) and brilliant green agar (BGA, Oxoid Ltd., Basingstoke, Hampshire, UK) with a 10 μL loop, and incubated at 37 °C during 24 h. Colonies with a typical *Salmonella* appearance (shiny pink with or without black centers and sometimes glossy large 3–5 mm in diameter on XLD agar or grey-reddish/pink and slightly convex on BGA) were collected and maintained on TSA slants at room temperature for further biochemical and serological testing. *Salmonella* cultures were subjected to Gram test or viscosity of potassium hydroxide test, oxidase test and reactions in triple sugar iron agar (TSI; Oxoid Ltd., Basingstoke, Hampshire, UK). Isolates with typical biochemical reactions of *Salmonella* were further confirmed with the API 20E system (BioMérieux, Marcy-ÍÉtoile, France) and slide agglutination test using *Salmonella* polyvalent O-antiserum (Difco, USA).

2.3.2.2. Water samples. The detection of *Salmonella* spp. was carried out analyzing a sample volume of 100 mL that was filtered through 0.45 μm-pore size membrane filters. The filter disks were aseptically placed on BPW and incubated at 37 °C for 18–24 h. The subsequent procedure was identical to the one described for clams and sediment.

2.3.3. Vibrio species

2.3.3.1. Clams and sediment samples. The qualitative *Vibrio* spp. analysis was performed according to the ISO/TS 21872-1 method (ISO, 2007) for *Vibrio parahaemolyticus* and *Vibrio cholerae*. Initially, 225 mL alkaline peptone water (APW; Oxoid Ltd., Basingstoke, Hampshire, UK) with pH 8.6 was added to 25 g of homogenized sample and incubated at 41.5 °C for 6 h. An additional enrichment was performed with 1 mL of the first enrichment and 10 mL of APW. This broth culture was incubated at 41.5 °C for 18 h. These enrichment cultures were plated on thiosulphate citrate bile salt sucrose agar (TCBS; Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 37 °C for 24 h. Five to ten green colonies, presumptively selected as *V. parahaemolyticus*, or yellow colonies, presumptively selected as *V. cholerae*, with 2–3 mm in diameter were isolated in TSA for identification. After incubation at 37 °C for 24 h, isolates were subjected to Gram stain, oxidase test and TSI reactions. Testing for sensitivity towards the Vibriostatic O-129 (2,4-Diamino-6,7-diisopropylpteridin-Phosphate) was performed using test discs with respectively 10 μg and 150 μg Vibriostatic O-129 (Oxoid Ltd., Basingstoke, Hampshire, UK) on TSA and incubated at 37 °C for 24 h. Further biochemical identification was done using the API 20NE system (micromethod tests for the identification of non-enteric Gram-negative rods; BioMérieux, Marcy-ÍÉtoile, France). All biochemically identified *V. cholerae* were tested by slide agglutination with polyvalent 01, nonspecific Ogawa and Inaba antisera (BD/Difco, Sparks, Maryland, USA).

For quantification of *Vibrio* spp., 25 g of homogenized sample was added to 225 mL of APW. Serial ten-fold dilutions of samples were prepared in APW and incubated on TCBS at 37 °C for 24 h. Five green and yellow characteristic colonies with 2–3 mm diameter from each TCBS Petri dish were subjected to Gram stain and

Table 2
Results of two-way ANOVA evaluating the effects of season and species (*Venerupis pullastra* and *Ruditapes philippinarum*) on TVC, *Escherichia coli* and *Vibrio* spp. levels.

	Clams				Water				Sediment			
	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*
TVC												
Season (S)	5	0.277	80.8	0.000*	5	0.463	187.8	0.000*	5	0.315	235.7	0.000*
Species (S)	1	4.42	1289.6	0.000*	1	2.29	928.7	0.000*	1	3.712	2775.3	0.000*
S × S	5	0.233	68.1	0.000*	5	0.091	37.0	0.000*	5	0.242	180.6	0.000*
Error	12	0.003			12	0.002			12	0.001		
Escherichia coli												
Season (S)	5	0.357	15.3	0.000*	5	2.02	871.1	0.000*	5	0.028	0.763	0.595
Species	1	14.8	634.1	0.000*	1	6.58	2841.8	0.000*	1	5.13	142.7	0.000*
S × S	5	0.312	13.4	0.000*	5	0.184	79.6	0.000*	5	0.055	1.53	0.257
Error	12	0.023			12	0.002			11	0.036		
Vibrio spp.												
Season (S)	5	1.52	107.2	0.000*	5	0.814	84.4	0.000*	5	2.86	195.5	0.000*
Species	1	1.07	75.6	0.000*	1	5.49	569.0	0.000*	1	5.96	407.4	0.000*
S × S	5	0.285	20.1	0.000*	5	0.628	65.1	0.000*	5	0.071	4.88	0.016*
Error	12	0.014			12	0.010			12	0.015		

Abbreviations: TVC, total viable counts. Asterisk indicates significant differences.

oxidase tests (bioMérieux, France). Gram-negative and oxidase-positive strains were then identified as *Vibrio* spp. Counts were expressed as log colony-forming units per gram of sample (log CFU g^{-1}).

2.3.3.2. Water samples. The detection of *Vibrio* spp. was carried out by analyzing 100 mL water filtered through 0.45 μm-pore size membrane filters. The filter disks were aseptically placed on APW and incubated at 41.5 °C for 6 h. A further enrichment was performed by employing 1 mL of the first enrichment and 10 mL of APW and posterior incubation at 41.5 °C for 18 h. These enrichment cultures were plated on TCBS and the following steps were identical to those described for clams and sediment. The quantification of *Vibrio* spp. was carried out by filtering 10 and 100 mL that through 0.45 μm-pore size membrane filters, and incubation on TCBS at 37 °C for 24 h. The identification and enumeration of colonies was identical to the procedure described for clams and sediment. Counts were expressed as log colony-forming units per gram of sample (log CFU mL^{-1}).

2.4. Statistical analysis

Two-way analysis of variance (ANOVA) was performed to detect the effects of season and species on TVC, *E. coli*, *Salmonella* spp. and *Vibrio* spp. levels. Additionally, one-way ANOVA was used to evaluate the differences between clams, water and sediment samples and also throughout the sampling year. Whenever necessary, data was transformed to satisfy normal distribution and homoscedasticity requirements. If transformed data could not meet these assumptions, non-parametric analysis of variance (Kruskall–Wallis) was performed, followed by non-parametric multiple comparison test (Dunn test). The correlation between bacteria concentration in clams, water and sediment and the environmental parameters (water temperature, salinity, pH and dissolved oxygen) were tested by Pearson's correlation coefficients. Differences were considered statistically significant at $p < 0.05$. All analyses were performed using the software STATISTICA™ 7.0 (Statsoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Physical–chemical parameters

Overall, the biggest amplitudes in the physical–chemical parameters were registered in Barreiro in the first 6-months (Table 1). Water temperature varied between 12.1 °C and 19.3 °C in Trafaria and 11.3 °C and 21.2 °C in Barreiro, with the highest temperatures in spring and summer and the lowest in winter. Salinity was relatively higher in Trafaria than in Barreiro, being higher in warmer months and lower in winter due to rain. Indeed, statistically significant correlations ($p < 0.05$) were obtained between rainfall and salinity in Trafaria ($r = -0.83$) and with

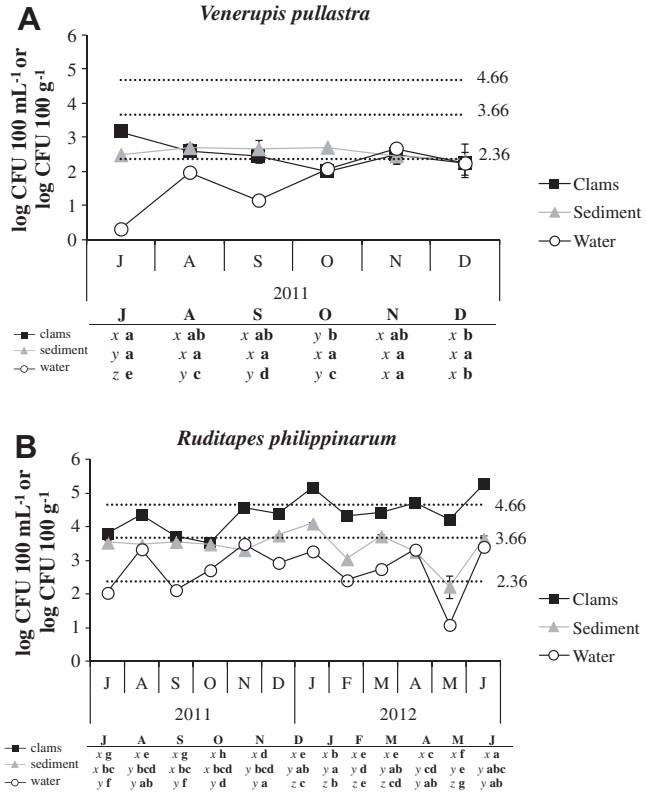


Fig. 2. Seasonal variations of *Escherichia coli* levels in (A) *Venerupis pullastra*, and (B) *Ruditapes philippinarum* clams, sediment (log CFU 100 g^{-1}) and harvesting waters (log CFU 100 mL^{-1}) from Trafaria and Barreiro. Values represent mean ± SD ($n = 3$). Different letters denote significant differences ($p < 0.05$) between samples (*x*–*z*) or between sampling months (a–h). For example, *y*b means that sample *y* is significantly different from samples *x*, *z*, and sampling month b was significantly different from all other sampling months. Reference lines indicate regulatory levels used for the classification of harvesting areas in the European Union (zones B, C and prohibited: 2.36; 3.66 and 4.66 log CFU/100 g).

temperature in Barreiro (r = -0.76; data not shown). The concentration of dissolved oxygen ranged between 7.05 mg L^{-1} (summer) and 9.13 mg L^{-1} (winter) in Trafaria and 5.19 mg L^{-1} (autumn) and 10.26 mg L^{-1} (summer) in Barreiro. The pH ranged between 8.165 (summer) and 8.424 (autumn) in Trafaria and 7.942 (summer) and 8.307 (autumn) in Barreiro. Rainfall ranged between 3 mm (summer) and 105 mm (autumn) (data not shown).

3.2. Seasonality and inter-specific differences

A similar trend was observed between both clam species for all bacteria, with a marked seasonal variation in all samples analyzed on TVC, *E. coli* and *Vibrio* spp. levels. The only exception occurred with *E. coli* in Trafaria sediment, where the levels did not significantly vary in the 6-months sampling period (Table 2; and Fig. 2A). Significantly higher TVC and *E. coli* levels were found during winter in water from both sampling sites (Figs. 2 and 3), whereas in sediment TVC and *E. coli* levels were higher in summer, except *E. coli* in *R. philippinarum* sediment that registered maximum levels in winter (Figs. 2 and 3). Concerning clams, *V. pullastra* showed significantly higher bacterial levels in autumn (for TVC) and summer (for *E. coli*), whereas *R. philippinarum* higher levels were present during winter (for TVC) and summer (for TVC and *E. coli*) (Figs. 2 and 3).

The seasonal trends of *Vibrio* spp. revealed significant higher levels in summer (*R. philippinarum* clams, water and sediment; and *V. pullastra* sediment) and autumn (*V. pullastra* clams and water) (Fig. 4). Generally, *Vibrio* spp. was not detectable during the winter months in both clam species, water and sediment samples from both sampling sites. Among *Vibrio* spp. strains assessed, *V. parahaemolyticus* was detected in all samples collected in warmer months (spring and summer), but only occasionally in colder months. *V. cholerae* non-O1 was detected in water samples of both sampling sites (17% and 25% in Trafaria and Barreiro, respectively) as well as in *R. philippinarum* clams (8%) and *V. pullastra* sediment (17%), whereas *V. cholerae* O1 was only detected in *R. philippinarum* surrounding water (October) (Table 3).

The occurrence of *Salmonella* spp. was mostly detected in clams (17% and 25% for *V. pullastra* and *R. philippinarum*, respectively) (Table 3). Indeed, in *R. philippinarum* sediment *Salmonella* spp. only occurred in December and April.

A significant influence of clam species was observed in all bacteria analyzed (Table 2), where significantly higher levels were generally found in *R. philippinarum*, as well as in its surrounding water and sediment throughout the year compared to *V. pullastra*. Additionally, a general pattern (sediment > clams > water) was observed for TVC and *Vibrio* spp. levels (Figs. 3 and 4), contrasting with *E. coli* counts in *R. philippinarum* (clams > sediment > water) and *V. pullastra* (clams = sediment > water, with a final general convergence in the cold months) (Fig. 2).

Pearson's correlation analysis revealed that the TVC in *R. philippinarum* clam had a significantly negative relationship (p < 0.05) with sediment (Table 4). In contrast, *Vibrio* spp. revealed a statistically positive correlation between both clam species and sediment (Table 4). Only *R. philippinarum* clam revealed a significantly positive correlation with water in *E. coli* and *Vibrio* spp. counts (Table 4). Also a positive significant relationship was observed between *R. philippinarum* surrounding water and sediment for *E. coli* and *Vibrio* spp.

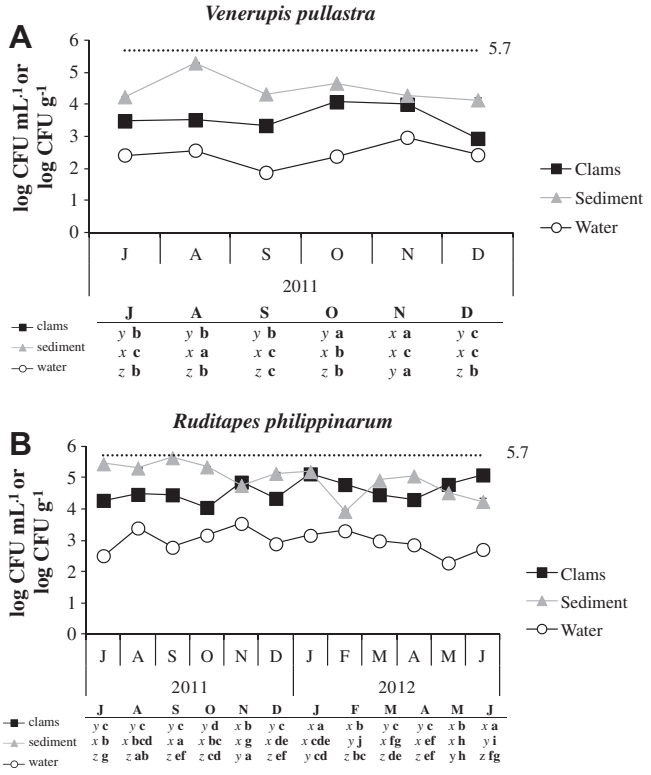


Fig. 3. Seasonal variations of TVC levels in (A) *Venerupis pullastra* and (B) *Ruditapes philippinarum* clams, sediment (log CFU g^{-1}) and harvesting waters (log CFU mL^{-1}) from Trafaria and Barreiro. Values represent mean ± SD (n = 3). Different letters denote significant differences (p < 0.05) between samples (x–z) or between sampling months (a–j). For example, yb means that sample y is significantly different from samples x, z, and sampling month b was significantly different from all other sampling months. Reference line indicate recommended limit (5.7 log CFU g^{-1}) for good quality fresh and frozen bivalve molluscs.

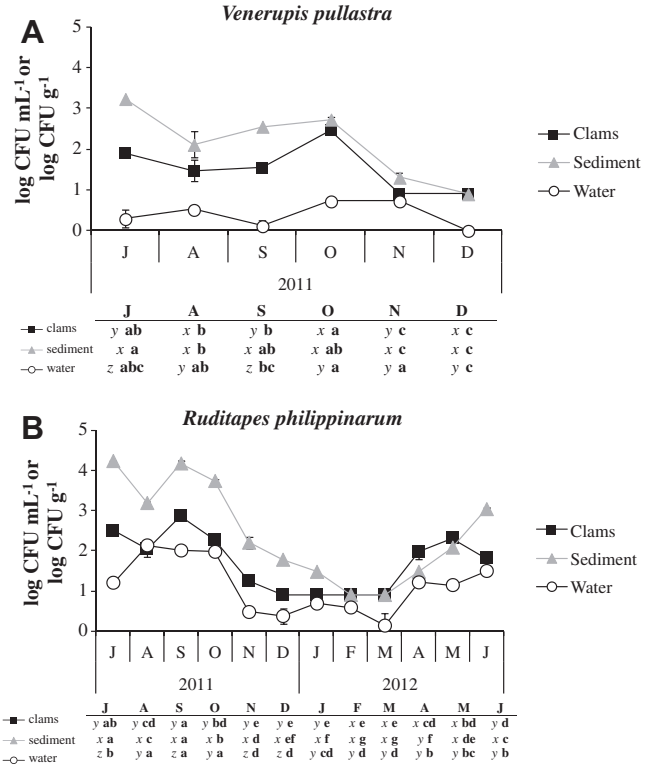


Fig. 4. Seasonal variations of *Vibrio* spp. levels in (A) *Venerupis pullastra* and (B) *Ruditapes philippinarum* ms, sediment (log CFU g^{-1}) and harvesting waters (log CFU mL^{-1}) from Trafaria and Barreiro. Values represent mean ± SD (n = 3). Different letters denote significant differences (p < 0.05) between samples (x–z) or between sampling months (a–g). For example, yb means that sample y is significantly different from samples x, z, and sampling month b was significantly different from all other sampling months.

Table 3
Occurrence of *Salmonella* spp. and *Vibrio* spp. in clams (*Venerupis pullastra*, VP, and *Ruditapes philippinarum*, RP), water and sediment from Trafaria and Barreiro over a one-year period.

		Clams		Water		Sediment	
		VP	RP	Trafaria	Barreiro	Trafaria	Barreiro
Salmonella spp.	*N*	1	3	0	0	0	2
	%	17	25	0	0	0	17
Vibrio parahaemolyticus	*N*	2	4	1	4	3	4
	%	33	33	17	33	50	33
Vibrio cholerae non-O1	*N*	0	1	1	3	1	0
	%	0	8	17	25	17	0
Vibrio cholerae O1	*N*	0	0	0	1	0	0
	%	0	0	0	8	0	0

Abbreviations: *N*, number; %, percentage frequency.

Table 4
Pearson correlation coefficients between clams, water and sediment samples in each pathogenic bacteria studied.

		VP			RP		
		Clam	Water	Sediment	Clam	Water	Sediment
TVC	Clam	1.00			1.00		
	Water	0.44	1.00		0.08	1.00	
	Sediment	0.26	0.11	1.00	−0.59*	−0.04	1.00
E. coli	Clam	1.00			1.00		
	Water	−0.53	1.00		0.58*	1.00	
	Sediment	0.08	−0.16	1.00	0.25	0.63*	1.00
Vibrio spp.	Clam	1.00			1.00		
	Water	0.33	1.00		0.80*	1.00	
	Sediment	0.86*	0.14	1.00	0.84*	0.78*	1.00

Abbreviations: TVC, total viable counts; *E. coli*, *Escherichia coli*; VP, *Venerupis pullastra*; RP, *Ruditapes philippinarum*.

Table 5
Pearson correlation coefficients between the environmental parameters and pathogenic bacteria levels.

	Clams						Water						Sediment					
	VP			RP			Trafaria			Bareiro			Trafaria			Barreiro		
	TVC	*E. coli*	*Vibrio* spp.	TVC	*E. coli*	*Vibrio* spp.	TVC	*E. coli*	*Vibrio* spp.	TVC	*E. coli*	*Vibrio* spp.	TVC	*E. coli*	*Vibrio* spp.	TVC	*E. coli*	*Vibrio* spp.
TVC	1.00			1.00			1.00			1.00			1.00			1.00		
E. coli	−0.23	1.00		0.78*	1.00		0.48	1.00		0.64*	1.00		0.69	1.00		0.43	1.00	
Vibrio spp.	0.38	−0.27	1.00	−0.37	−0.58	1.00	0.56	0.53	1.00	−0.15	−0.08	1.00	0.24	−0.40	1.00	0.65*	0.26	1.00
Temperature	0.40	−0.35	0.84*	−0.19	−0.10	0.78*	−0.45	−0.27	0.38	−0.63**	−0.22	0.69*	0.61	0.04	0.78	0.20	−0.21	0.68*
Salinity	0.09	0.67	0.43	0.01	0.12	0.05	−0.22	−0.87*	−0.14	−0.56	−0.38	0.02	0.00	−0.68	0.85*	−0.29	−0.33	−0.21
D.O.	0.06	0.15	−0.29	0.19	−0.05	−0.09	0.85*	0.37	0.26	−0.01	−0.22	−0.42	0.17	0.54	−0.44	0.12	−0.04	−0.08
pH	0.46	−0.21	−0.50	−0.05	−0.26	−0.30	0.83**	0.85*	0.57	0.29	−0.28	−0.57	−0.05	0.44	−0.71	−0.10	−0.46	−0.40
Rainfall	0.17	−0.42	−0.48	0.05	0.12	−0.70*	0.36	0.77	0.22	0.59*	0.32	−0.65*	−0.44	0.26	−0.78	−0.11	0.13	−0.50

Abbreviations: TVC, total viable counts; *E. coli*, *Escherichia coli*; D.O., dissolved oxygen; VP, *Venerupis pullastra*; RP, *Ruditapes philippinarum*.

3.3. Influence of environmental parameters on bacterial levels

Statistical analyses only showed significant positive relationships between TVC and *E. coli* (in *R. philippinarum* and in its surrounding water) or *Vibrio* spp. (in sediment of *R. philippinarum* sampling site) (Table 5).

Concerning the correlations between bacteria flora and the environmental parameters, TVC revealed a significant positive correlation with dissolved oxygen and pH in *V. pullatra* surrounding water, and with rainfall in *R. philippinarum* surrounding water, whereas negative correlation was detected between TVC and temperature in *R. philippinarum* surrounding water. *E. coli* levels only showed significant correlations in *V. pullastra* surrounding water with salinity (negative) and pH (positive). Finally, *Vibrio* spp. was positively correlated with salinity in *V. pullastra* sediment, and with temperature in both clam species, and in *R. philippinarum* surrounding water and sediment. In contrast, rainfall was statistically negative correlated with *Vibrio* spp. in *R. philippinarum* and its surrounding water (Table 5).

4. Discussion

Many factors are involved in the distribution and survival of microorganisms in estuarine ecosystems, including biotic and abiotic parameters of surrounding seawater (Rippey, 1994). Ecosystems such as estuaries tend to be naturally eutrophic, with concentrated river water entering coastal sites and supplying nutrients that may support the occurrence and spread of microbial contamination (Janelidze et al., 2011). The high TVC and *E. coli* levels found in clams, particularly in *R. philippinarum*, harvested in Tagus estuary reflect the quality of the surrounding waters that

are influenced by environmental parameters, such as temperature, salinity, rainfall patterns, and by anthropogenic activities in the basin. Like in most estuaries, Tagus is affected by storm water runoff, livestock production, wildlife, sewer overflows and recreational ports, constituting important sources of faecal contamination in bivalve harvesting areas (Garreis, 1994). Several industries and farming explorations located nearby Tagus estuary, combined with a highly populated area, contribute to the production of heavily polluted wastewaters. This fact might explain the higher contamination levels detected in Barreiro compared to Trafaria. The present study revealed that *E. coli* concentrations in *R. philippinarum* were positively correlated with bacterial levels in water, which is consistent with other field surveys performed on levels of *E. coli* in mussels (Plusquellec et al., 1990; Solic et al., 1999). However, no correlations were identified for *V. pullastra*. Furthermore, the amount of water filtered by bivalves is strongly influenced by inter- and intra-specific variations, ranging between twenty and one hundred litres per day (Robertson, 2007). This means that bivalves feeding physiology determines the accumulation of pathogenic microorganisms filtered from the overlying water (Burkhardt and Calci, 2000). Several studies demonstrated that clearance rates are different between the two clam species: in *R. philippinarum* can range between 8 and 20 L/g^{-1} per h (Nakamura, 2004) and in *V. pullastra* can range between 1 and 3 L/g^{-1} per h (Beiras et al., 1993). These phenomena may partially explain seasonal and geographical differences in microbial content of bivalves (Hernroth et al., 2002).

Overall, this study corroborates findings of several authors, which have demonstrated that seasonality and environmental parameters (e.g., rainfall or dry weather periods, water temperature, and salinity) are the main factors influencing bacterial concentration in bivalves (Farias et al., 2010; Sasikumar and Krishnamoorthy, 2010; Derolez et al., 2012). Several authors reported a positive link between freshwater input and survival rate of faecal counts in areas (Strickland and Parsons, 1968; Raveendran et al., 1990). Yet, in this study such correlation was only detected in water surrounding *V. pullastra*. Nonetheless, significantly higher TVC and *E. coli* levels were found in winter months in water and sediment from both sites. Davies et al. (1995) reported that in estuarine waters faecal coliforms in sediment can be released to the water column in rough weather events leading to increased coliform levels in water. The increase in suspended solids and turbidity in coastal waters can also contribute to survival of faecal bacteria by providing an organic substrate as well as protection from light in addition to a mechanism for transport downstream (Pommepuy et al., 1992).

Concerning to the current legislation, TVC levels recorded in both clams' species harvested in Tagus estuary were lower than the recommended limit (5.7 log CFU g^{-1}; Fig. 3) for good quality fresh and frozen bivalve molluscs given by International Commission on Microbiological Specifications for Foods (ICMSF, 1986). However, both clam species revealed high *E. coli* levels, well above the legal limit (2.36 log CFU 100 g^{-1} the equivalent to 230 CFU 100 g^{-1} of edible meat). The higher levels commonly found in the exotic clam throughout the year demonstrated that this clam was collected in C areas, in which bivalves can be collected and commercialized only after relaying a long period, a practice that is uncommon among clam fisherman's and marketers in Tagus estuary. However, in some periods of the year *E. coli* levels reached peaks (January and June 2012), corresponding a prohibited areas for harvesting bivalves. In contrary, the *E. coli* levels found in native clams corresponded mostly to category B, requiring depuration before marketing, but in October and December 2011 the levels were equivalent as category A. Though there is a potential health hazard associated with the consumption of clams without depuration or improper cooking time *Salmonella* spp. was generally isolated when higher levels of indicators of faecal pollution (*E. coli*) were detected, particularly in *R. philippinarum*. Similar findings were detected with other bivalve species, like oysters (Spino 1966; Lear and Jaworski, 1969; Wilson and Moore, 1996; Farias et al., 2010). It has also been demonstrated (Van Donsel and Geldreich, 1971; Hendricks, 1972) and substantiated in this study that the frequency of occurrence of *Salmonella* spp. is generally higher in sediment samples compared with water samples. From the public health point of view, the current legislation requires the total absence of *Salmonella* spp. in 25 g of bivalves to ensure safe products. Therefore, in this study, clams were not considered to be safe for human consumption.

Temperature, salinity, rainfall and dissolved oxygen have been highlighted as the major significant drivers of *Vibrio* seasonality (Thompson et al., 2003; Baffone et al., 2006). The current study confirmed that Vibrios are more frequently detected when seawater temperature increases, particularly *V. parahaemolyticus* and *V. cholerae*, suggesting that the risk is greatest in summer months, whereas its density declined gradually towards winter (low water temperature and higher rainfall) in clams, water and sediment. Research carried out by various authors on bivalves has also shown the presence of *V. parahaemolyticus* in summer months (Cioglia et al., 1982; Cavallo and Stabili, 2002). Several authors also observed a positive correlation between the occurrence of *Vibrio* spp. and temperature in bivalves species, such as mussels (Ripabelli et al., 1999; Lhafi and Kühne, 2007) and oysters (De Paola et al., 2003). In this study, negative correlation was also found between the occurrence of *Vibrio* spp. and rainfall in *R. philippinarum* clams and surrounding water, which is in accordance with findings of Neumann et al. (1972). Salinity also plays an important role in the occurrence of *Vibrio* spp., particularly *V. cholerae*, in coastal and estuarine regions (Barbieri et al., 1999; Jiang, 2001). Indeed, *V. cholerae* optimal growth conditions occur at lower salinities (<25 mg L^{-1}) (Singleton et al., 1982). In this study, *V. cholerae* was mostly detected in *R. philippinarum* sampling site that showed lower salinity (average 34 mg L^{-1}) than Trafaria (36 mg L^{-1}; Table 3). Cavallo and Stabilli (2002) also reported that a mean salinity value of 34.76 mg L^{-1} is compatible with the occurrence of *V. cholerae*.

Generally, the abundance of all bacteria were higher in the sediment than in the water column, which has also been reported in other studies (Haller et al., 2009; Nagvenkar and Ramaiah, 2009). Van Donsel and Geldreich (1971) and Goyal et al. (1977) noticed that sediments appear to provide the most conducive ecological niche for the survival of pathogens. This high bacterial load in sediments may be due to enhanced survival by low/no exposure to stressors, such as sunlight and predation, or by increased availability of nutrients (Craig et al., 2002).

The lack of correlation between faecal indicators of pollution and *Vibrio* species shows that they are not useful indicators for the presence of these bacteria in seawater and marine foods. Similar findings were obtained by Thompson et al. (1976), Hackney et al. (1980) and Kiiyukia et al. (1989). Several *Vibrio* strains present a public health hazard as they are responsible for several illnesses (otitis, pharyngitis, wound infections) as well as foodborne gastroenteritis (De Paola et al., 1990; Mizunoe et al., 2000). Since the current European legislation does not specify limits for this organism, preventive measures should be implemented for *Vibrio* spp., taking into account that they are indigenous of estuarine and coastal regions and are normal constituents of mollusc flora, and are resistant to the depuration process (Desmarchelier, 2000; Barile et al., 2009). Presently, the only way to protect consumers against contamination with pathogenic *Vibrio* strains is to recommend proper cooking of clams before consumption.

This is the first study demonstrating the influence of environmental parameters on bacterial levels in bivalves, harvesting

waters and sediment from Tagus estuary. Overall, TVC and *E. coli* levels were higher in samples collected in colder months, which revealed higher dissolved oxygen, pH and rainfall, as well as lower temperature and salinity. In contrast, higher *Vibrio* spp. concentrations were detected in warmer months, where lower rainfall levels and higher salinity and temperature were found. This study also highlights the vulnerability of bivalve production areas to microbial contamination in a constantly changing environment. Additionally, it is also particularly useful for authorities to develop monitoring strategies of coastal microbiological contamination, and to adapt legislation in order to incorporate new microbial strains such as *Vibrio* spp. Indeed, this species can represent a danger to public health in the future, particularly in warmer periods that are expected to be more frequent due to climate change. Ultimately, the strategy used in this study will enable to estimate more accurate risks associated with the consumption of bivalves to human health by authorities. Education on the importance of depuration as a means of bivalves decontamination should be also incorporated into the general fishery management, in order to ensure safe product for consumers.

Acknowledgments

This study was supported by the SHELLPLANT research project (Ref. 232273) funded by the European Commission (FP7). The authors gratefully acknowledge to Jorge Camarão Jerónimo, Sandro Pinto and Pedro Patacas for their support in samples collection. The first author acknowledges a PhD scholarship of the Portuguese Foundation for Science and Technology (FCT; Ref. SFRH/BD/69274/2010), whereas the last two authors were supported by a research contract from the Ciência 2008 Program of FCT.

References

Almeida, C., Soares, F., 2012. Microbiological monitoring of bivalves from the Ria Formosa Lagoon (south coast of Portugal): a 20 years of sanitary survey. Mar. Pollut. Bull. 64, 252–262.

Baffone, W., Tarsi, R., Pane, L., Campana, R., Repetto, B., Mariottini, G.L., Pruzzo, C., 2006. Detection of free-living and plankton-bound vibrios in coastal waters of the Adriatic Sea (Italy) and study of their pathogenicity-associated properties. Environ. Microbiol. 8, 1299–1305.

Barbieri, E., Falzano, L., Fiorentini, C., Pianitti, A., Baffone, W., Fabbri, A., Matarrese, P., Caisere, A., Katouli, M., Kühn, I., Mölby, F., Bruscolini, F., Donelli, G., 1999. Occurrence, diversity, and pathogenicity of halophilic *Vibrio* spp. and non-O1 *Vibrio cholerae* from estuarine waters along the Italian Adriatic Coast. Appl. Environ. Microbiol. 65, 2748–2753.

Barile, N.B., Scopa, M., Nerone, E., Mascilongo, G., Recchi, S., Cappabianca, S., Antonetti, L., 2009. Study of the efficacy of closed cycle depuration system on bivalve molluscs. Vet. Ital. 45 (4), 555–566.

Beiras, R., Pérez-Camacho, A., Albentosa, M., 1993. Influence of food concentration on energy balance and growth performance of *Venerupis pullastra* seed reared in an open-flow system. Aquaculture 116, 353–365.

Brogueira, M.J., Cabeçadas, G., 2006. Identification of similar environmental areas in Tagus estuary by using multivariate analysis. Ecol. Indic. 6, 508–515.

Burkhardt, W., Calci, K.R., 2000. Selective accumulation may account for shellfish associated viral illness. Appl. Environ. Microbiol. 66 (4), 1375–1378.

Campos, C.J.A., Cachola, R.A., 2007. Faecal coliforms in bivalve harvesting areas of the Alvor lagoon (southern Portugal): influence of seasonal variability and urban development. Environ. Monit. Assess. 133, 31–41.

Cavallo, R.A., Stabili, L., 2002. Presence of vibrios in seawater and *Mytilus galloprovincialis* (Lam.) from the Mar Piccolo of Taranto (Ionian Sea). Water Res. 36, 3719–3726.

Cioglia, A.M., San, M., Uccheddu, A.M., 1982. Reperto di vibrioni alofili nelle acque litoranee e lagunari di Cagliari e nei prodotti ittici (molluschi e pesci) derivanti da esse o in vendita nei mercati della citt"a. Ig Mod. 77, 199–211.

Cook, D.W., 1991. Indicators and alternate indicators of growing waters. In: Ward, D.R., Ackney, C.H. (Eds.), Microbiology of Marine Food Products. AVI Book, Van Nostrand Reinhold, New York, pp. 19–39.

Craig, D.L., Fallowfield, H.J., Cromar, N.J., 2002. Enumeration of fecal coliforms from recreational coastal sites: evaluation of techniques for the separation of bacteria from sediments. J. Appl. Microbiol. 93, 557–565.

Davies, C.M., Long, J.A.H., Donald, M., Ashbolt, N.J., 1995. Survival of faecal microorganisms in marine and freshwater sediments. Appl. Environ. Microbiol. 61 (5), 1888–1896.

De Paola, A., Hopkins, L.H., Peeler, J.T., Wentz, B., McPhearson, R.M., 1990. Incidence of *Vibrio parahaemolyticus* in US coastal waters and oysters. Appl. Environ. Microbiol. 8, 2299–2302.

De Paola, A., Nordstrom, J.L., Bowers, J.C., Wells, J.G., Cook, D.W., 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Appl. Environ. Microbiol. 69 (3), 1521–1526.

Deepanjali, A., Kumar, H.S., Karunasagar, I., Karunasagar, I., 2005. Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the Southwest Coast of India. Appl. Environ. Microbiol. 71 (7), 3575–3580.

Derolez, V., Soudant, D., Fiandrino, A., Cesmat, L., Serais, O., 2012. Impact of weather conditions on *Escherichia coli* accumulation in oysters of the Thau lagoon (the Mediterranean, France). J. Appl. Microbiol. 114, 516–525.

Desmarchelier, P.M., 2000. Vibrio. In: Robinson, R., Batt, C., Patel, P. (Eds.), Encyclopedia of Food Microbiology. Academic Press, USA, pp. 2237–2242.

EC, 2001. Opinion of the scientific committee on veterinary measures relating to public health on vibrio vulnificus and vibrio parahaemolyticus (in raw and undercooked seafood). In: European Commission Health & Consumer Protection Directorate General. Directorate C—Scientific Opinions, pp. 1–64.

EC, 2004. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Off. J. Eur. Union L226, 83–127.

EC, 2007. Commission Regulation (EC) 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union L322, 1–18.

Eja, M.E., Abriba, C., Etok, C.A., Ikpeme, E.M., Arikpo, G.E., Enyi-Idoh, K.H., Ofor, U.A., 2008. Seasonal occurrence of Vibrios in water and shellfish obtained from the Great Kwa River Estuary, Calabar, Nigeria. Bull. Environ. Contam. Toxicol. 81, 245–248.

FAO, 2012. The State of World Fisheries and Aquaculture 2012. Food and Agriculture Organization of the United Nations, Rome, Italy, p. 209.

Farias, M.F., Rocha-Barreira, C.A., Carvalho, F.C.T., Silva, C.M., Reis, E.M.F., Costa, R.A., Vieira, R.H.S.F., 2010. Microbiological conditions of *Tagelus plebeius* (Lightfoot, 1786) (Mollusca: Bivalvia: Solecurtidae) and the water in the Ceará river estuary in Fortaleza – CE. Bol. Inst. Pesca 36 (2), 135–142.

Gameiro, C., Brotas, V., 2010. Patterns of phytoplankton variability in the Tagus Estuary (Portugal). Estuar. Coast. 33, 311–323.

Garreis, M.J., 1994. Sanitary surveys of growing waters. In: Hackney, C.R., Pierson, M.D. (Eds.), Environmental Indicators and Shellfish Safety. Chapman & Hall, New York, pp. 289–330.

Goyal, S.M., Gerba, C.P., Melnick, J.L., 1977. Occurrence and distribution of bacterial indicators and pathogens in canal communities along the Texas coast. Appl. Environ. Micro. 34, 139–149.

Hackney, C.R., Ray, B., Speck, M.L., 1980. Incidence of *Vibrio parahaemolyticus* in and the microbiological quality of seafood in North Carolina. J. Food Protect. 43, 769–773.

Hahn, M.W., 2006. The microbial diversity of inland water. Curr. Opin. Biotechnol. 17, 256–261.

Haller, L., Pote, J., Loizeau, J.L., Wildi, W., 2009. Distribution and survival of faecal indicator bacteria in the sediment of the Bay of Vidy, Lake Geneva, Switzerland. Ecol. Indic. 9 (3), 540–547.

Hendricks, C.W., 1972. Enteric bacterial growth rates in river water. Appl. Microbiol. 24, 168–174.

Hernroth, B.E., Conden-Hansson, A.-C., Rehnstam-Holm, A.-S., Girones, R., Allard, A.K., 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Appl. Environ. Microbiol. 68 (9), 4523–4533.

ICMSF, 1986. Microorganisms in Foods 2. Sampling for Microbiological Analysis: Principles and Specific Applications. University of Toronto Press, second ed. Blackwell Scientific Publications, Toronto.

ISO, 2000. ISO 9308-1:2000. Water quality — detection and enumeration of *Escherichia coli* and coliform bacteria. — Part 1: Membrane filtration method, p. 10.

ISO, 2001. ISO 16649-2:2001. Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* – Part 2: colony-count technique at 44 C using 5-bromo-4-chloro-3-indolyl β-D-glucuronide, p. 16.

ISO, 2002. ISO 6579:2002. Microbiology of food and animal feeding stuffs — horizontal method for the detection of Salmonella spp., p. 34.

ISO, 2007. ISO/TS 21872-1:2007. Microbiology of food and animal feeding stuffs — horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. — Part 1: detection of *Vibrio parahaemolyticus* and *Vibrio cholerae*, p. 26.

Janelidze, N., Jaiani, E., Lashkhi, N., Tskhvediani, A., Kokashvili, T., Gvarishvili, T., Jgenti, D., Mikashavidze, E., Diasamidze, R., Narodny, S., Obiso, R., Whitehouse, C.A., Huq, A., Tediashvili, M., 2011. Microbial water quality of the Georgian coastal zone of the Black Sea. Mar. Pollut. Bull. 62, 573–580.

Jiang, S.C., 2001. *Vibrio cholerae* in recreational beach waters and tributaries of Southern California. Hydrobiologia 460, 157–164.

Kiiyukia, C., Venkateswaran, K., Navarro, I.M., Nakano, H., Kawakami, H., Hasiimoto, H., 1989. Seasonal distribution of *Vibrio parahaemolyticus* serotypes along the oyster beds in Hiroshima coast. J. Fac. Appl. Biol. Sci. 28, 49–61.

Lear, D.W., Jr., Jaworski, N.A., 1969. Sanitary bacteriology of the Upper Potomac estuary. In: Technical Report No. 6, Chesapeake Technical Support Laboratory, Federal Water Pollution Control Administration, Department of Interior, Washington, DC.

Lees, D., 2000. Viruses and bivalve shellfish. Int. J. Food Microbiol. 59 (1–2), 81–116.

Lees, D., Younger, A., Dore, B., 2010. Depuration and relaying. In: Rees, G., Pond, K., Kay, D., Bartram, J., Santo Domingo, J. (Eds.), Safe Management of Shellfish and Harvest Waters. World Health Organization (WHO), IWA Publishing, London, p. 37.

Lhafi, S.K., Kühne, M., 2007. Occurrence of *Vibrio* spp. in blue mussels (*Mytilus edulis*) from the German Wadden Sea. Int. J. Food Microbiol. 116, 297–300.

Lindstrom, E.S., 2001. Investigating influential factors on bacterioplankton community composition: results from a field study of five mesotrophic lakes. Microbiol. Ecol. 42, 598–605.

Martinez-Urtaza, J., Saco, M., De Novoa, J., Perez-Piñeiro, P., Peiteado, J., Lozano-Leon, A., Garcia-Martin, O., 2004. Influence of environmental factors and human activity on the presence of *Salmonella* serovars in a marine environment. Appl. Environ. Microbiol. 70 (4), 2089–2097.

Mizunoe, Y., Wai, S.N., Ishikawa, T., Takade, A., Yoshida, S., 2000. Resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under starvation. FEMS Microbiol. Lett. 186, 115–120.

Nagvenkar, G.S., Ramaiah, N., 2009. Abundance of sewage-pollution indicator and human pathogenic bacteria in a tropical estuarine complex. Environ. Monit. Assess. 155, 245–256.

Nakamura, Y., 2004. Suspension feeding and growth of juvenile Manila clam *Ruditapes philippinarum* reared in the laboratory. Fish. Sci. 70, 215–222.

Neumann, D.A., Benemon, H., Hubster, E., Thi Nhu Tuan, N., Tie-Van, L., 1972. Vibrio parahaemolyticus in the Republic of Vietnam. Am. J. Trop. Med. Hyg. 22, 464–470.

Oliveira, J., Cunha, A., Castilho, F., Romalde, J.L., Pereira, M.J., 2011. Microbial contamination and purification of bivalve shellfish: crucial aspects in monitoring and future perspectives e a mini-review. Food Control 22, 805–816.

Plusquellec, A., Beucher, M., Prieur, D., Le Gal, Y., 1990. Contamination of the mussel, *Mytilus edulis* Linnaeus, 1758, by enteric bacteria. J. Shellfish Res. 9, 95–101.

Pommepuy, M., Guillaud, J.F., Dupray, E., Derrien, A., Le Guyader, F., Cormier, M., 1992. Enteric bacteria survival factors. Water Sci Technol. 12, 93–103.

RASFF, 2012. The Rapid Alert System for Food and Feed (RASFF) – Annual Report 2011. Publications Office of the European Union, p. 47.

Raveendran, O., Gore, P.S., Iyer, T.S.G., Varma, P.R.G., Sankaranarayanan, V.N., 1990. Occurrence of enteric bacteria in seawater and mussels along the southwest coast of India. Indian J. Mar. Sci. 19, 282–284.

Reilly, A., Käferstein, F., 1997. Food safety hazards and the application of the principles of hazard analysis and critical control point (HACCP) system for their control in aquaculture production. Aquacult. Res. 28, 735–752.

Ripabelli, G., Sammarco, M.L., Grasso, G.M., Fanelli, I., Caprioli, A., Luzzi, I., 1999. Occurrence of *Vibrio* and other pathogenic bacteria in *Mytilus galloprovincialis* (mussels) harvested from Adriatic Sea, Italy. Int. J. Food Microbiol. 49, 43–48.

Rippey, S.R., 1994. Infections diseases associated with molluscan shellfish consumption. Clin. Microbiol. Rev. 7, 419–425.

Robertson, L.J., 2007. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: a review. Int. J. Food Microbiol. 120 (3), 201–216.

Sasikumar, G., Krishnamoorthy, M., 2010. Faecal indicators and sanitary water quality of shellfish-harvesting environment: influences of seasonal monsoon and river-runoff. Indian J. Geol. Mar. Sci. 39 (3), 434–444.

Singleton, F.L., Attwell, R., Jangi, S., Colwell, R., 1982. Influence of salinity and organic nutrient concentration on survival and growth of *Vibrio cholerae* in aquatic microcosms. Appl. Environ. Microbiol. 43, 1080–1085.

Solic, M., Krstulovic, N., Jozic, S., Curac, D., 1999. The rate of concentration of faecal coliforms in shellfish under different environmental conditions. Environ. Int. 25, 991–1000.

Spino, D.F., 1966. Elevated-temperature technique for the isolation of *Salmonella* from streams. Appl. Microbiol. 14, 591–595.

Strickland, J.D.H., Parsons, T.R., 1968. A practical handbook of seawater analysis. Bull. Fish. Res. Board Can. 167, 311.

Thompson, C.A., Vanderzant, C., Ray, S.M., 1976. Relationship of *Vibrio parahaemolyticus* in oysters, water and sediment and bacteriological indices. J. Food Sci. 41, 117–122.

Thompson, J.R., Randa, M.A., Marcelino, L.A., Tomita-Mitchell, A., Lim, E., Polz, M.F., 2003. Diversity and dynamics of a North Atlantic coastal *Vibrio* community. Appl. Environ. Microbiol. 70, 4103–4110.

Van Donsel, D.J., Geldreich, E.E., 1971. Relationship of *Salmonella* to faecal coliforms in bottom sediments. Water Res. 5, 1079–1087.

Vernocchi, P., Maffei, M., Lanciotti, R., Suzzi, G., Gardini, F., 2007. Characterization of Mediterranean mussels (*Mytilus galloprovincialis*) harvested in Adriatic Sea (Italy). Food Control 18 (12), 1575–1583.

Wilson, I.G., Moore, J.E., 1996. Presence of *Salmonella* spp. and *Campylobacter* spp. in shellfish. Epidemiol. Infect. 116, 147–153.

Yannarell, A.C., Triplett, E.W., 2004. Within and between lake variability in the composition of bacterioplankton communities: investigations using multiple spatial scales. Appl. Environ. Microbiol. 70, 214–223.

CHAPTER 4

Microbiological responses to depuration and transport of native and exotic clams at optimal and stressful temperatures

Anacleto P., Maulvault A. L., Chaguri M., Pedro S., Nunes M. L., Rosa R. and Marques A.

(2013)

Food Microbiology, 36(2): 365–373
DOI: 10.1016/j.fm.2013.07.002

Food Microbiology 36 (2013) 365–373

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Microbiological responses to depuration and transport of native and exotic clams at optimal and stressful temperatures

Patrícia Anacleto [a,b], Ana Luísa Maulvault [a], Milena Chaguri [b], Sónia Pedro [a], Maria Leonor Nunes [a], Rui Rosa [c], António Marques [a,*]

[a] Division of Aquaculture and Upgrading (DivAV), Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.), Avenida de Brasília, 1449-006 Lisbon, Portugal
[b] Aquaculture Center, São Paulo State University (UNESP), Via de Acesso Prof. Paulo Donato Castellane s/n, 14884-900 Jaboticabal, São Paulo, Brazil
[c] Guia Marine Laboratory, Center of Oceanography, Faculty of Sciences, University of Lisbon (FCUL), Campo Grande, 1749-016 Lisbon, Portugal

ARTICLE INFO

Article history:
Received 30 January 2013
Received in revised form
1 July 2013
Accepted 9 July 2013
Available online 19 July 2013

Keywords:
Depuration
Transport
Clams
Escherichia coli
Vibrio spp.
Total viable counts

ABSTRACT

The microbiological responses of two bivalves species from Tagus estuary, *Venerupis pullastra* (native clam) and *Ruditapes philippinarum* (exotic clam) were investigated during 48 h of depuration and subsequent simulated transport in semi-dry conditions at two temperatures (4 and 22 °C) until reaching 50% lethal time (LT50). Regardless of temperature and species, the maintenance of clams in water for 48 h (depuration period) did not affect LT50 during transport. *R. philippinarum* showed higher survival rates than *V. pullastra*, always reaching LT50 later, especially at 4 °C. Significant differences between clams' species were found in almost all microbiological parameters. This can be related with clams' biological activity and habitat environmental conditions since both clams do not coexist in Tagus estuary. Depuration was efficient to reduce the bacterial load, particularly *Escherichia coli*, but not efficient to remove *Vibrio* spp. In both species, the growth of *Vibrio* spp. was inhibited at 4 °C, whereas exponential growth occurred at 22 °C. Total viable counts significantly increased in most treatments, while *E. coli* counts significantly decreased to undetected levels, except for non-depurated *R. philippinarum* simulated transported at 4 °C. Thus, this study highlights the importance of clams depuration for at least 24 h in polluted estuarine areas, followed by transport at low temperatures (4 °C).

1. Introduction

The southern European exploitation of bivalve molluscan shellfish is mainly focused in coastal ecosystems, where this activity is of great social and economical importance (Berthou et al., 2005). Among marine bivalves, pullet carpet shell clam (*Venerupis pullastra*) and Japanese carpet shell clam (*Ruditapes philippinarum*) are the most important exploited bivalve molluscs. In Portugal, clams represent around 26% (5276 tonnes; FAO, 2012a) of molluscan production and Tagus estuary is one of the key clams' harvesting places. *V. pullastra* is a native species of Tagus estuary that usually occurs in sandy and muddy-sand sediments from the low tide mark (lower shore) to a depth of 40 m (circalittoral; Macedo et al., 1999). On the other hand, *R. philippinarum* is an allochthonous species introduced in the beginning of 1970s for commercial exploitation/cultivation in North European Atlantic and Mediterranean coastal waters (Delgado and Pérez-Camacho, 2007; Flassch and Leborgne, 1992; Gosling, 2002; Jensen et al., 2004). The relevance of this exotic species in Portugal, particularly in Tagus estuary, has rapidly increased in the last decade, mostly due to its high adaptability and tolerance to environmental variations (e.g. salinity, temperature), dispersion capacity and ability to live at high animal densities (Gaspar, 2010).

Tagus estuary is subjected to strong anthropogenic pressures that affect water quality and result in high levels of faecal and pathogenic microorganisms responsible for many diseases (Lemarchand et al., 2004). Since they are filter-feeding organisms, bivalve molluscs inhabiting such environments can accumulate pathogenic microorganisms, such as bacteria, human viruses and microalgae, representing a significant health risk if consumed raw or lightly cooked (Cook, 1991; Lees et al., 2010). Generally, bivalves' bacterial composition is dominated by Gram-negative bacteria like Vibrionaceae and Enterobacteriaceae, with some of them being responsible for foodborne diseases (e.g. *Escherichia coli*, *Salmonella*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*; Cao et al., 2009). The current European regulations to ensure bivalve molluscs

* Corresponding author. Tel.: +351 213 027 025; fax: +351 213 015 948.
E-mail addresses: amarques@ipma.pt, marques_am@yahoo.com (A. Marques).

0740-0020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fm.2013.07.002

microbiological safety (Regulation EC Nos. 853/2004 and 854/2004; EC, 2004a, 2004b) enforces the need to depurate bivalves with microbial contaminants below 4600 CFU *E. coli* per 100 g of flesh and inter-valvar fluid (areas classified as category B). Generally, a depuration period of 24–48 h is efficient to reduce *E. coli* and *Vibrio* spp. in mussels, depending on the initial level of contamination and bivalve' species (Barile et al., 2009; Cozzi et al., 2009). However, several fishermen trade non-depurated bivalves with wholesalers and retailers without sanitary control, particularly *R. philippinarum*. Along the trade chain these specimens are often subjected to wide temperature variations and not maintained in refrigerated conditions.

Several studies have been carried out to evaluate the effect of depuration in physiological and microbiological aspects of some clams' species, such as *Ruditapes decussates*, *Venerupis senegalensis*, *Chamelea gallina* and *Mercenaria mercenaria* (El-Shenawy, 2004; Howard et al., 2003; Maffei et al., 2009; Timoney and Abston, 1984). Yet, no studies were performed with *V. pullastra*, and as far as *R. philippinarum* is concerned only the physiological effect of temperature was assessed during aerial exposure, but not at a microbiological point of view (Ali and Nakamura, 1999, 2000; Ali et al., 1999).

In this context, the main objective of this study was to compare, for the first time, the microbiological responses (*E. coli*, *Vibrio* spp. and total viable counts – TVC) of two clam species, the native *V. pullastra* and the exotic *R. philippinarum* from Tagus estuary in two critical steps of their trade chain, i.e. depuration (48 h) and subsequent simulated transport in semi-dry conditions at two temperatures (optimal: 4 °C; stressful: 22 °C) until reaching 50% lethal time (LT50).

2. Materials and methods

2.1. Collection of samples and treatments

Two clam species were collected in the Summer of 2011 from two fishing areas: pullet carpet shell clam (*V. pullastra*) from Trafaria, located in the mouth of Tagus estuary, and Japanese carpet shell clam (*R. philippinarum*) from Alcochete, located in the upstream part of the estuary (Fig. 1). Tagus estuary is one of the largest estuaries of the European west coast, located in the most populated area of Portugal, with a broad shallow Bay covering an area of about 320 km^2 (Brogueira and Cabeçadas, 2006). Both clams were immediately stored under aseptic conditions in sterile plastic bags at 4 °C and transported in portable cooling chamber with temperature controller (WAECO CoolFreeze CF-40, Emsdetten, Germany) until their arrival to the laboratory (approximately two hours of transport period).

Specimens biometric data was obtained (mean ± standard deviation; $n = 100$) for *V. pullastra* (weight: 9.5 ± 3.0 g; shell length: 35.4 ± 4.1 mm; shell width: 17.1 ± 2.1 mm; shell height: 23.4 ± 2.9 mm) and *R. philippinarum* (weight: 17.6 ± 5.7 g; shell length: 40.5 ± 4.2 mm; shell width: 21.5 ± 2.8 mm; and shell height: 29.7 ± 3.2 mm). In total, 2264 animals were used (1180 native clams and 1084 exotic clams). Each species was divided in two portions: one being depurated during 48 h and subjected to simulated transport conditions in semi-dry conditions at optimal temperature conditions (4 °C; treatment DT4) or at stressful conditions (22 °C; treatment DT22) until reaching the time required for 50% mortality (LT50); the other portion of clams were only subjected to simulated transport conditions without depuration in semi-dry conditions at the same optimal (4 °C; treatment T4) or stressful conditions (22 °C; treatment T22) until reaching LT50. Temperature controlled chambers set at 4 or 22 °C were used to simulate transport, where clams packed in 1 kg net bags were placed inside plastic boxes with draining holes and covered with wet cloth. Thermometers and data-loggers were used to monitor temperature levels inside the chambers. Since clams are sensitive to physical damage, the animals were carefully handled to minimize mortalities. Daily, mortality was registered in each treatment and twenty-five specimens per treatment were randomly collected and measured for behavioural activity using the criteria described by El-Shenawy (2004) with some modifications (Table 1). Per treatment and sampling day, 15 animals were used for microbiological assessment. All analyses were carried out in triplicate.

2.1.1. Depuration experiment

Depuration was performed 2 h after clams collection and was conducted in two recirculated tanks (length, 31 cm and height, 40 cm) containing 15 L of UV sterilized seawater. The flow rate entering the circuit was 2.9 L/min. Each tank contained 2 kg of clams, which corresponds to clam densities usually employed at

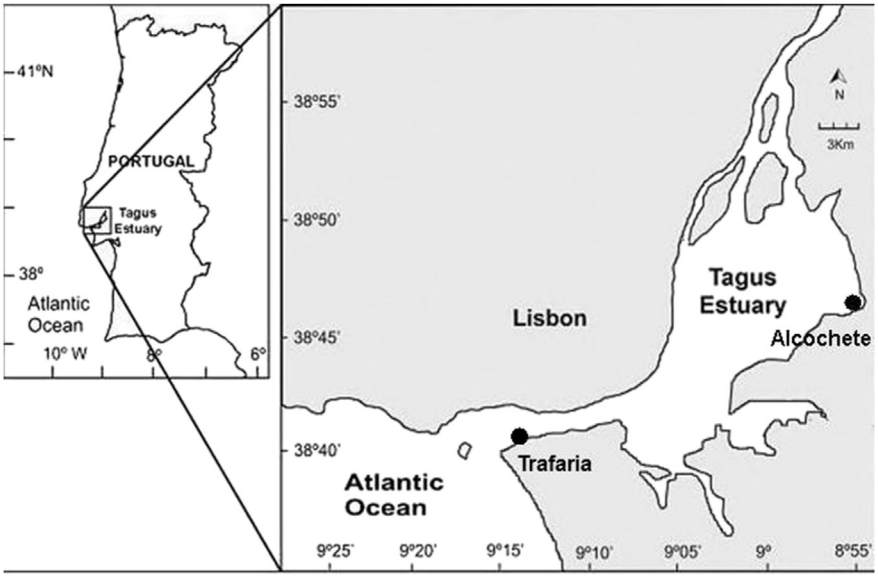


Fig. 1. Map of Portugal with the enlarged portion of Tagus estuary. Dark filled circles indicate the samplings sites (Trafaria for *V. pullastra* and Alcochete for *R. philippinarum*).

Table 1
Summary of criteria used for scoring behavioural clam activity.

Score	Criterion
0	Shell valve closed
1	Shell valve merely opened but mantle edge not clearly visible
2	Shell valve opened and mantle edge clearly visible
3	Shell valve opened and siphons extending up to half their full length
4	Shell valve opened and both siphons and foot protruding (i.e. dead clam)

Portuguese depuration facilities. Clams were not fed during the experimental period and seawater was kept with continuous aeration and at constant temperature (18.3 °C), salinity (35.5 g/L) and pH (6.98). Bivalve samples were collected at 0, 24 and 48 h for further analyses.

2.2. Microbiological analyses

Clams were aseptically washed and shucked with a sterile knife. Then, the flesh and intravalvular fluid were extracted aseptically.

2.2.1. E. coli and total viable counts

The enumeration of *E. coli* was carried out according to the ISO 16649-2 (ISO, 2001) and the pour plate method was used for total viable counts (TVC). Edible clam meat was initially diluted in Maximum Recovery Diluent (MRD; Oxoid Ltd., Basingstoke, Hampshire, UK) and homogenized during 60 s in a Stomacher 400 (Seward Laboratory System, London, UK). Appropriate serial decimal dilutions were performed in MRD followed by culture on tryptone bile X-glucuronide agar (Chromocult TBX; Merck, Darmstadt, Germany), followed by an initial incubation step at 37 ± 1 °C during 4.0 ± 0.5 h (for stressed cells) and a second incubation step at 44 ± 1 °C for 18 ± 2 h, for quantification of *E. coli*. TVC was assessed on trypticase soy agar (TSA; Merck, Darmstadt, Germany) and incubated at 22 °C for 5 days. After incubation, Petri dishes with 30–300 colonies were chosen to count blue colonies typical from β-glucuronidase positive *E. coli*. Microbiological counts were expressed as log colony-forming units per gram of sample (log CFU/g) for TVC and CFU/100 g for *E. coli*.

2.2.2. Vibrio spp.

For quantification of *Vibrio* spp., clam meat was added to alkaline peptone water (APW). APW included 10 g/L NaCl (Merck, Darmstadt, Germany) and 10 g/L bacteriological peptone (Oxoid Basingstoke, Hampshire, UK), adjusted to pH 8.6 ± 0.2 with 1 M NaOH. Serial ten-fold dilutions of samples were prepared in APW and plated on thiosulphate citrate bile salt sucrose agar (TCBS; Oxoid, Basingstoke, Hampshire, UK) and incubated at 37 °C for 24 h. All green and yellow colonies were presumed *Vibrio* spp. (Cheesborough, 1991). Five green and yellow characteristic colonies with 2–3 mm diameter from each TCBS Petri dish were subjected to Gram stain and oxidase tests (bioMérieux, France). Gram-negative and oxidase-positive strains were then identified as *Vibrio* spp. Counts were expressed as log colony-forming units per gram of sample (log CFU/g).

2.3. Statistical analysis

Two-way analysis of variance (ANOVA) was performed to detect significant differences in TVC, *E. coli* and *Vibrio* spp. levels between clams species and simulated transport temperature (4 and 22 °C). Additionally, one-way ANOVA was used to evaluate the effect of depuration and transport time in each treatment. Whenever necessary, data was transformed to satisfy normal distribution and homoscedasticity requirements. If transformed data could not meet these assumptions, non-parametric analysis of variance (Kruskal–Wallis) was performed, followed by non-parametric multiple comparison test (Dunn test). Correlations between the studied microbiological parameters were analyzed for each treatment with Pearson's correlation coefficients for linear correlation. Differences were considered statistically significant at $p < 0.05$. All analyses were performed using the software STATISTICA™ 7.0 (Statsoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Mortality rate

Fig. 2 shows the changes in cumulative mortality rate of native and exotic clams during depuration and simulated transport, or only during simulated transport without depuration. During depuration, cumulative mortality was very low (0–3%) and no significant differences were found between clams' species. A significant difference in mortality of both species was observed during simulated transport of depurated ($F = 10.5$, $p = 0.004$) and non-depurated clams ($F = 12.8$, $p = 0.002$; Table 2). *V. pullastra* always revealed higher mortality rate than *R. philippinarum*. These differences were found after 48 h simulated transport at both temperatures for depurated clams (Fig. 2A), while for non-depurated clams such differences were observed after 96 and 48 h simulated transport at 4 and 22 °C, respectively (Fig. 2B).

Additionally, the increase in mortality was always faster in both clams exposed to higher temperatures (22 °C) reaching LT50 more rapidly (72 h for *V. pullastra* and 96 h for *R. philippinarum*; Fig. 2) than at lower temperatures (4 °C; 120 h for *V. pullastra* and 336 h for *R. philippinarum*) for depurated ($F = 8.3$, $p = 0.001$) and non-depurated clams ($F = 4.6$, $p = 0.043$) (Table 2). Such differences were only observed after 72 h of simulated transport in both species subjected to depuration (Fig. 2A), while for non-depurated clams were found after 48 h of transport in *V. pullastra* and 96 h of transport in *R. philippinarum* (Fig. 2B).

3.2. Microbiological analyses

3.2.1. TVC levels

In the beginning of the experiment, TVC levels were significantly higher in *V. pullastra* (4.9 log CFU/g) than *R. philippinarum* (3.9 log CFU/g; Fig. 3). A significant influence of species was only observed in depurated clams ($F = 8.9$, $p = 0.007$; Table 2), but not in non-depurated clams. Higher TVC was generally registered in *V. pullastra* compared to *R. philippinarum*. During depuration, TVC levels significantly decreased in both clams species (0.6 and 0.9 log CFU/g, respectively), whereas during simulated transport a significant increase in TVC was detected in almost all treatments, except in depurated *V. pullastra* at 4 °C (Fig. 3).

Concerning transport temperatures, significantly higher TVC levels were observed for both clam species at 22 °C than at 4 °C. A significant influence of temperature was found in depurated ($F = 57.6$, $p < 0.001$; during all simulated transport in both species), and non-depurated clams ($F = 7.6$, $p = 0.012$; after 24 h of simulated transport in *R. philippinarum* and 48 h of simulated transport in *V. pullastra*) (Table 2; Fig. 3). For *R. philippinarum*, TVC showed a significant positive linear relationship with mortality and *Vibrio* spp. in all treatments (Table 3), while for *V. pullastra* a significant positive linear relationship was found in both treatments at 22 °C with mortality and *Vibrio* spp. and only with mortality in non-depurated clams and simulated transported at 4 °C (T4; Table 4).

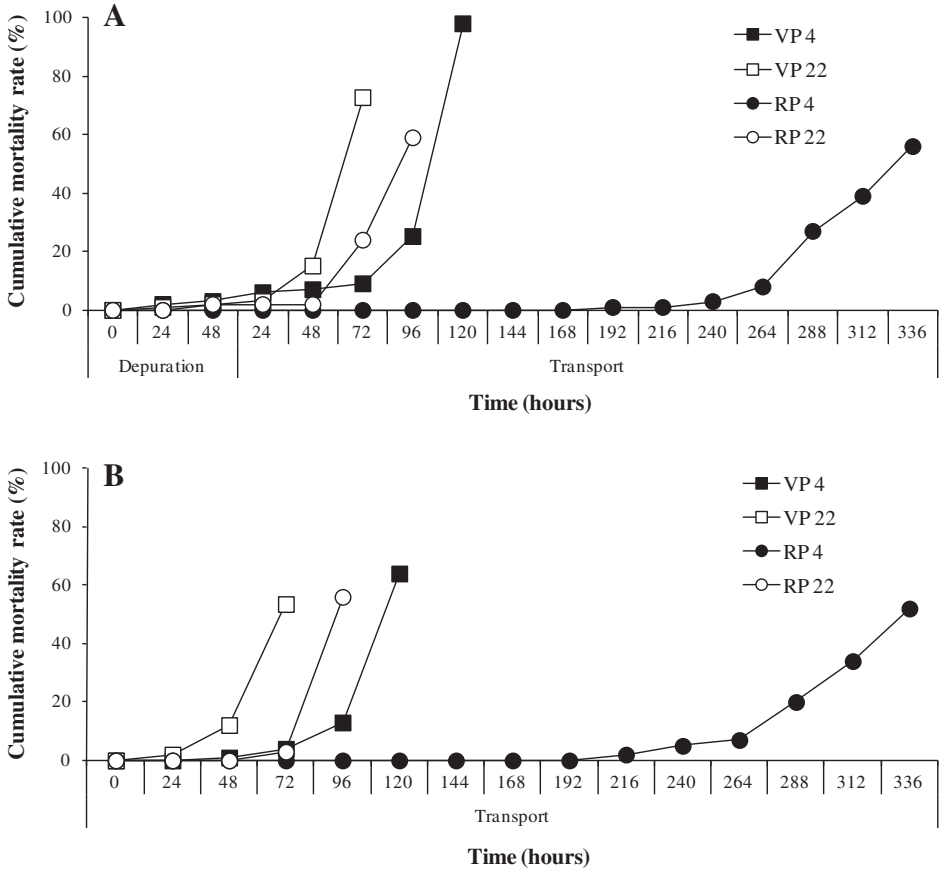


Fig. 2. Cumulative mortality rate (%) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry simulated transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry simulated transport at 4 or 22 °C.

3.2.2. *E. coli* levels

The levels of *E. coli* in depurated and non-depurated clam species transported in semi-dry conditions at 4 and 22 °C are reported in Fig. 4. In the beginning of the experiment, *E. coli* levels were significantly higher in *R. philippinarum* (2033 CFU/100 g) than in *V. pullastra* (794 CFU/100 g). Nevertheless, immediately after 24 h of depuration significant reductions of *E. coli* counts occurred in both clam species, reaching undetected levels until the end of the experiment at both temperatures (Fig. 4A). A significant influence of species ($F = 20.1$, $p < 0.001$) was found in non-depurated clams (Table 2), where *R. philippinarum* showed significantly higher *E. coli* levels than *V. pullastra* during simulated transport at 4 °C and until 48 h simulated transport at 22 °C (Fig. 4B). Regarding transport temperature, significant lower values were only found with non-depurated clams ($F = 4.4$, $p = 0.048$; Table 2) after 48 h of simulated transport (Fig. 4B).

Despite the *E. coli* levels in non-depurated native clams significantly decreased immediately after 24 h simulated transport, undetected levels only occurred at the end of simulated transport at 22 °C, but not at 4 °C. In exotic clams, a decrease of *E. coli* levels was also observed during simulated transport, though the reduction was more pronounced at 22 °C. *E. coli* levels showed a significant negative correlation with *Vibrio* spp. only in depurated *V. pullastra* transported at 4 °C (DT4; Table 4), while a significant negative linear relationship between *E. coli* and TVC was found only in T22 treatment of *R. philippinarum* (Table 3).

Table 2
Results of two-way ANOVA evaluating the effects of simulated transport temperature (4 and 22 °C) and species (*Venerupis pullastra* and *Ruditapes philippinarum*) on cumulative mortality, TVC, *Escherichia coli* and *Vibrio* spp. levels.

	Depurated				Non-depurated			
	df	MS	*F*	*p*	df	MS	*F*	*p*
Cumulative mortality								
Temperature (T)	1	22.6	8.3	0.001*	1	6.4	4.6	0.043*
Species (S)	1	28.6	10.5	0.004*	1	17.8	12.8	0.002*
T × S	1	0.056	0.020	0.887	1	2.5	1.8	0.197
Error	20	2.7			20	1.4		
TVC								
Temperature (T)	1	31.0	57.6	<0.001*	1	7.4	7.6	0.012*
Species (S)	1	4.8	8.9	0.007*	1	1.2	1.2	0.277
T × S	1	0.198	0.367	0.551	1	0.010	0.011	0.919
Error	20	0.538			20	0.971		
Escherichia coli								
Temperature (T)	1	0.371	2.5	0.132	1	0.859	4.4	0.048*
Species (S)	1	0.371	2.5	0.132	1	3.9	20.1	<0.001*
T × S	1	0.371	2.5	0.132	1	0.322	1.7	0.212
Error	20	0.151			20	0.194		
***Vibrio* spp.**								
Temperature (T)	1	26.4	42.4	<0.001*	1	19.7	11.2	0.003*
Species (S)	1	5.6	9.0	0.007*	1	0.610	0.349	0.561
T × S	1	0.015	0.025	0.876	1	0.101	0.058	0.812
Error	20	0.622			20	1.75		

Abbreviations: TVC, total viable counts. Asterisk indicates significant differences.

3.2.3. *Vibrio spp. levels*

Similarly to TVC, *Vibrio* spp. levels in the beginning of the experiment were significantly higher ($p = 0.007$) in *V. pullastra* (2.6 log CFU/g) compared to *R. philippinarum* (1.8 log CFU/g; Fig. 5). A significant influence of species was only observed in depurated

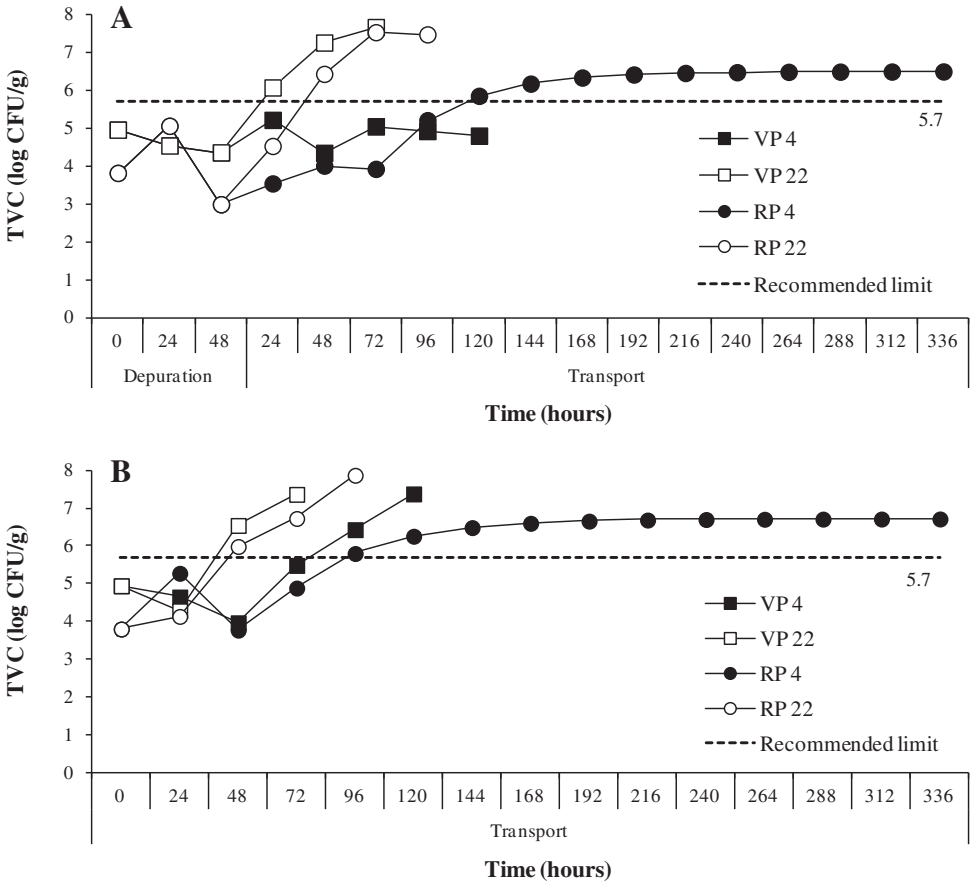


Fig. 3. TVC levels (log CFU/g) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry simulated transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry simulated transport at 4 or 22 °C. The dashed line indicates the recommended limit. Abbreviations: TVC, total viable counts. Values represent mean ± SD ($n = 3$).

Table 3
Pearson correlation coefficients between cumulative mortality, TVC, *Escherichia coli* and *Vibrio* spp. levels in each treatment of *Ruditapes philippinarum*.

	Mortality	TVC	*E. coli*	*Vibrio* spp.
DT4				
Mortality	1.00			
TVC	0.747 (0.001)*	1.00		
E. coli	−0.137 (0.614)	−0.101 (0.709)	1.00	
Vibrio spp.	0.845 (<0.001)*	0.838 (<0.001)*	−0.438 (0.090)	1.00
DT22				
Mortality	1.00			
TVC	0.676 (0.004)*	1.00		
E. coli	−0.215 (0.423)	−0.295 (0.267)	1.00	
Vibrio spp.	0.806 (<0.001)*	0.904 (<0.001)*	−0.425 (0.101)	1.00
T4				
Mortality	1.00			
TVC	0.723 (0.018)*	1.00		
E. coli	−0.409 (0.240)	−0.268 (0.455)	1.00	
Vibrio spp.	0.831 (0.003)*	0.882 (0.001)*	−0.292 (0.413)	1.00
T22				
Mortality	1.00			
TVC	0.679 (0.044)*	1.00		
E. coli	−0.465 (0.208)	−0.840 (0.005)*	1.00	
Vibrio spp.	0.678 (0.045)*	0.938 (<0.001)*	−0.654 (0.056)	1.00

Abbreviations: TVC, total viable counts, DT4 and DT22, depurated and transported clams at 4 or 22 °C, T4 and T22, non-depurated clams transported at 4 or 22 °C. *p*-values are indicated in brackets and an asterisk indicates a statistically significant correlation.

Table 4
Pearson correlation coefficients between cumulative mortality, TVC, *Escherichia coli* and *Vibrio* spp. levels in each treatment of *Venerupis pullastra*.

	Mortality	TVC	*E. coli*	*Vibrio* spp.
DT4				
Mortality	1.00			
TVC	0.154 (0.584)	1.00		
E. coli	−0.166 (0.554)	0.293 (0.289)	1.00	
Vibrio spp.	0.204 (0.465)	−0.489 (0.064)	−0.599 (0.018)*	1.00
DT22				
Mortality	1.00			
TVC	0.815 (0.001)*	1.00		
E. coli	−0.251 (0.408)	−0.192 (0.529)	1.00	
Vibrio spp.	0.819 (0.001)*	0.920 (<0.001)*	−0.461 (0.113)	1.00
T4				
Mortality	1.00			
TVC	0.769 (0.016)*	1.00		
E. coli	−0.154 (0.692)	−0.084 (0.830)	1.00	
Vibrio spp.	0.054 (0.889)	0.519 (0.152)	−0.312 (0.413)	1.00
T22				
Mortality	1.00			
TVC	0.762 (0.047)*	1.00		
E. coli	−0.518 (0.233)	−0.549 (0.202)	1.00	
Vibrio spp.	0.684 (0.090)*	0.989 (<0.001)*	−0.598 (0.157)	1.00

Abbreviations: TVC, total viable counts, DT4 and DT22, depurated and transported clams at 4 or 22 °C, T4 and T22, non-depurated clams transported at 4 or 22 °C. *p*-values are indicated in brackets and an asterisk indicates a statistically significant correlation.

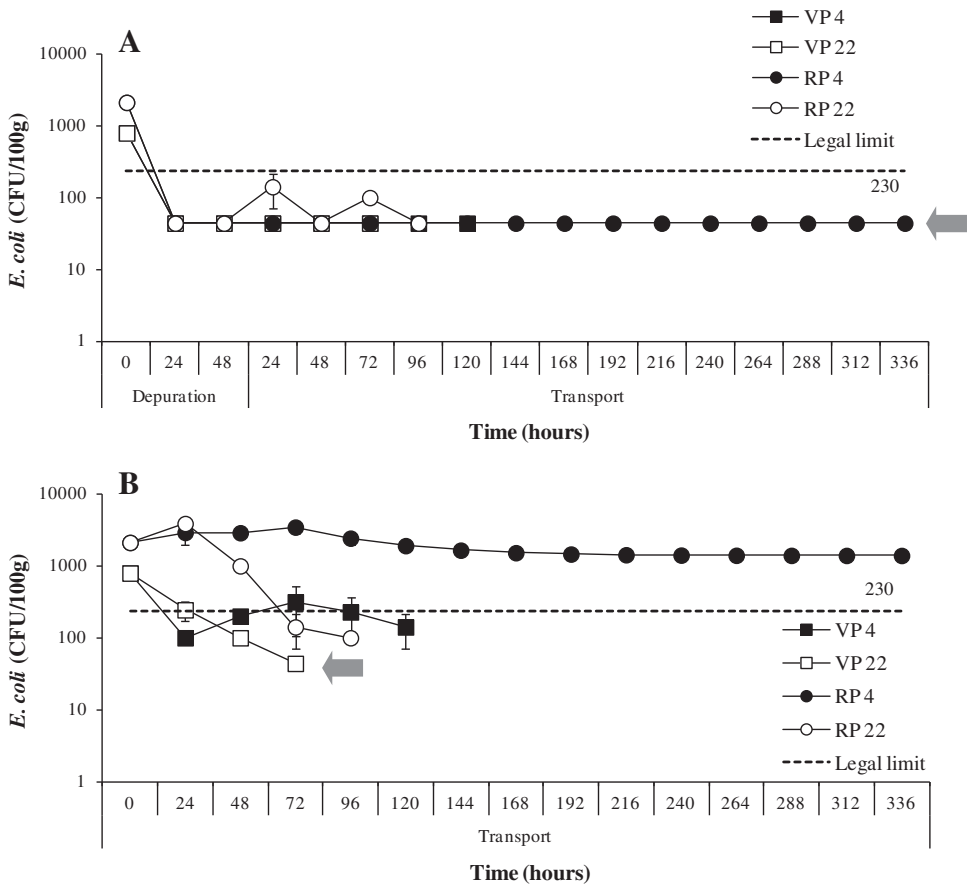


Fig. 4. *Escherichia coli* levels (CFU/100 g) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry simulated transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry simulated transport at 4 or 22 °C. The dotted line indicates the legal limit and the grey arrow indicates the detection limit. Values represent mean ± SD ($n = 3$).

clams ($F = 9.0$, $p = 0.007$; Table 2), where significant higher levels were generally found in *V. pullastra* throughout depuration and both transport temperatures (Fig. 5). During depuration, *Vibrio* spp. concentration significantly increased in native clams ($p = 0.001$), reaching 3.6 CFU/g, while in exotic clams the increase was not significant, attaining 2.4 CFU/g (Fig. 5A).

A significant influence of transport temperature in both species was always observed in depurated ($F = 42.4$, $p < 0.001$) and non-depurated clams ($F = 11.2$, $p = 0.003$) (Table 2; Fig. 5). The levels of *Vibrio* spp. significantly increased in both clams' species transported at 22 °C, attaining higher levels in exotic clams (7.5–7.9 CFU/g) compared to native clams (6.2–6.3 CFU/g) at LT50. Furthermore, depurated and non-depurated native clams transported at 4 °C, did not reveal significant changes in *Vibrio* spp. values throughout simulated transport, whereas exotic clams revealed a significant increase in *Vibrio* spp. levels (2.0 and 2.8 CFU/g, respectively). *Vibrio* spp. revealed a significant positive correlation with mortality and TVC in all treatments of *R. philippinarum* (Table 3). In contrast, *Vibrio* spp. in *V. pullastra* was statistically positively correlated with TVC and mortality in DT22 and T22, and negatively correlated with *E. coli* in DT4 (Table 4).

4. Discussion

4.1. Clam mortality

The long survival of *R. philippinarum* exposed to air and maintained at low temperatures is related to the exotic species' higher adaptability and tolerance to environmental changes (Ali and Nakamura, 1999, 2000; Ali et al., 1999; Uzaki et al., 2003). The extended survival of *R. philippinarum* compared to *V. pullastra* can be attributed to distinct physiological conditions that enable different adaptations to habitats in Tagus estuary. *R. philippinarum* usually burrows in sand, muddy gravel or stiff clay, below mid-tide level up to 4 m deep and is usually distributed in the upstream part of the estuary, where they are subjected to large variations of environmental parameters (CIESM, 2012; Jones et al., 1993). In contrast, *V. pullastra* can be found in deeper waters up to 40 m, in the mouth of the Tagus estuary and burying 5 cm in the sediment, where specimens are subjected to low variations of environmental parameters (FAO, 2012b; Macedo et al., 1999).

Overall, regardless of transport temperature, the use of depuration enabled additional 48 h storage extension of both clam species until attaining LT50, which has practical and economical interest for marketing strategies. The results also showed that semi-dry simulated transport at 4 °C was able to prolong 48 and 240 h the survival of *V. pullastra* and *R. philippinarum*, respectively compared to 22 °C. This occurred because at chilled temperatures the metabolism of clams is reduced, with lower oxygen demands and reduced production of excretion products. The growth of microorganisms is also inhibited as well as enzymatic deterioration reactions (Sea Grant, 2012).

4.2. Clam microbiology

Both clam species harvested in Tagus estuary revealed high *E. coli levels*, well above the legal limit (230 CFU/100 g of wet meat), thus confirming as being from B category harvesting areas,

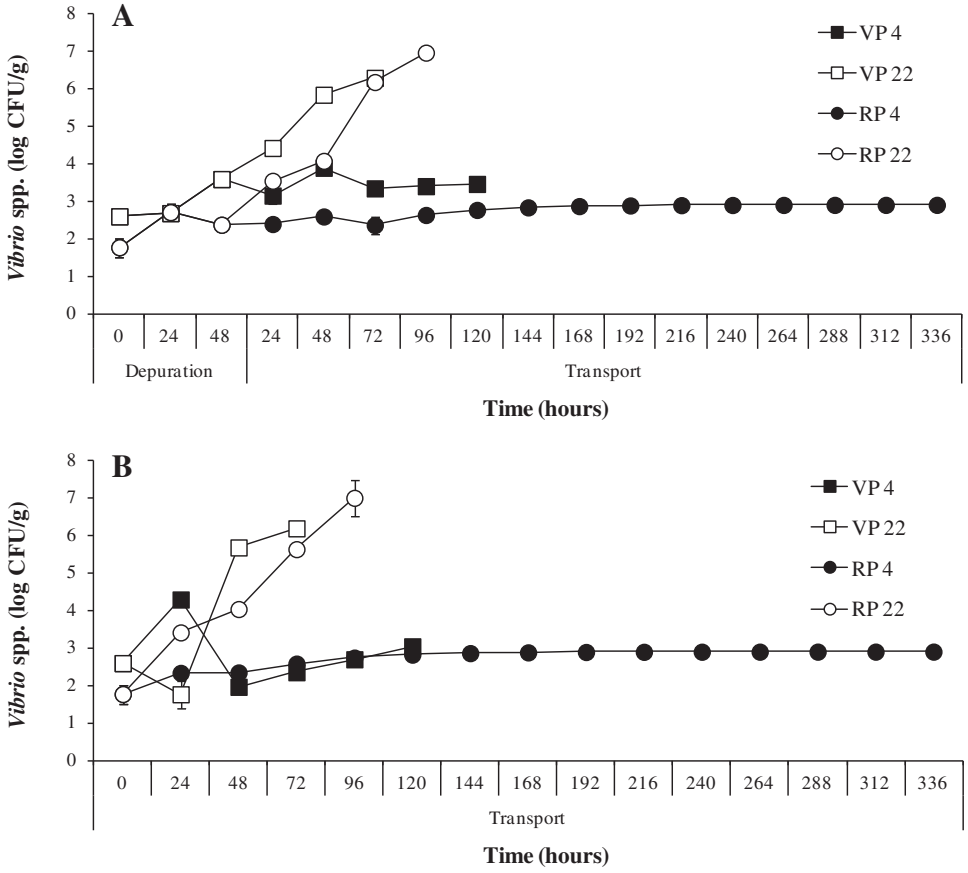


Fig. 5. *Vibrio* spp. levels (log CFU/g) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry simulated transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry simulated transport at 4 or 22 °C. Values represent mean ± SD ($n = 3$).

requiring depuration in a purification centre or relaying over a period to meet the health standards before commercialization for human consumption according to the current regulations (EC, 2004a, 2004b). *E. coli* levels were higher in exotic clams, which reflect higher pressures from wastewater treatment effluents, industrial or animal explorations and urbanization in the area of Alcochete (according to the National Information System of Water Resources (SNIRH) in Portugal), where *R. philippinarum* was collected.

TVC recommended limit for good quality fresh and frozen bivalve molluscs (5.7 log CFU/g; ICMSF, 1986) was reached in all treatments with both clam species, except depurated *V. pullastra* transported at 4 °C. This increase in TVC counts may indicate physiological and biochemical changes in bivalves' tissues (Bernárdez and Pastoriza, 2011). In fact, the proliferation of certain bacterial groups can occur when the defence system is weakened, namely by hypoxic/anoxic stress (De Zwaan et al., 2002).

Vibrio spp. are naturally occurring bacteria in estuarine and marine waters and their density are positively correlated with seawater temperature (DePaola et al., 2003; Duan and Su, 2005; Kaneko and Colwell, 1973; Marino et al., 2005). In this study, the initial higher levels of *Vibrio* spp. and TVC found in *V. pullastra* compared to *R. philippinarum* reflected warmer seawater temperature in Trafaria than in Alcochete during the sampling day (despite of the greater influence of oceanic waters in the former location).

All microbiological parameters varied with transport temperature. Indeed, both clam species transported in optimal conditions (4 °C) revealed: i) constant levels of *Vibrio* spp., ii) TVC increased at slow rates and iii) *E. coli* decreased at slow rates. In contrast, clams transported in stressful conditions (22 °C) generally showed: i) increased *Vibrio* spp. and TVC levels and ii) decreased *E. coli* (always at fast growth rates). The growth of *Vibrio* spp. in shellfish seems to be inhibited at chilling temperatures, whereas they can rapidly multiply once exposed to elevated temperatures (>25 °C; Gooch et al., 2002). Clams' biological activity is also affected by environmental thermal conditions (Sobral and Widdows, 1997) and, therefore, filtration activity, enzymatic activity, clearance rate, respiration rate and excretion rate generally increase in warmer environments (Brock et al., 1986; Loosanoff, 1953).

In general, the depuration process was particularly efficient in the reduction of TVC and *E. coli* levels in both clam species, but not with *Vibrio* spp. Several studies have also shown the effectiveness of depuration in the elimination of *E. coli* in other clam species, such as *Chamelea gallina* (Barile et al., 2009) and *Egeria radiate* (Ekanem and Adegoke, 1995). In contrast, other studies reported the efficiency of depuration to reduce *Vibrio* spp. counts in other clam species (*Paphia undulata*) (El-Gamal, 2011). In fact, *Vibrio* spp. requires longer depuration periods than *E. coli* to become effective (Cozzi et al., 2009; Croci et al., 2002; Lopez-Joven et al., 2011). Croci et al. (2002) indicated that 44 h of depuration process led a decline of *Vibrio* by a factor of only 10, whereas Cozzi et al. (2009) describes that 72 h reduced *Vibrio* contamination to level close or below the detection limit of the methods. Also, Lopez-Joven et al. (2011) reported that at least 10 days depuration at 20 °C is effective to reduce *Vibrio* load. However, bivalve quality is reduced with time due to the lack of feed in the depuration process, thus representing significant economic losses to stakeholders when depuration periods above 48 h are implemented. The lack of efficiency of depuration to

reduce *Vibrio* levels might be due to the colonization of *Vibrio* spp. in bivalves' intestinal tracts (Colwell and Liston, 1960; Eyles and Davey, 1984; Martínez et al., 2009; Su and Liu, 2007; Vasconcelos and Lee, 1972). Since depuration is not efficient to eliminate *Vibrio* spp., including dangerous foodborne strains like *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, bivalve transport at higher temperatures and the increase in coastal water temperatures due to climate changes might exacerbate the health risks to bivalve consumers in the near future.

The present study clearly demonstrates that *E. coli* is an insufficient microbiological indicator to evaluate the quality and healthiness of shellfish, namely *V. pullastra* and *R. philippinarum*, not only because it does not reflect the survival and distribution trends of vibrios and viruses in shellfish, but is also unable to demonstrate the general depuration capacity (Ripabelli et al., 1999). Furthermore, the current European legislation does not specify limits for *Vibrio* spp. (EC, 2001). Therefore, preventive measures should be implemented for *Vibrio* spp., taking into account that they are naturally found in seawater and normal constituents of mollusc flora (Barile et al., 2009) and some strains (such as *V. parahaemolyticus*) are the major cause of epidemics associated with the consumption of bivalves (Mead et al., 1999; Wittman and Flick, 1995). Presently, the only ways to protect consumers against contamination with pathogenic *Vibrio* strains is to recommend cooking clams before eating and to transport specimens in chilled conditions.

5. Conclusion

This is the first study where a comparison was made between two clam species, *V. pullastra* (native) and *R. philippinarum* (exotic) from Tagus estuary to assess their microbiological responses to depuration and simulated transport at optimal and stressful temperatures. This work also confirms the higher tolerance of the exotic clam species compared to native populations during aerial exposure. Regardless of temperature and species, the maintenance of clams in water for 48 h (depuration period) does not affect LT50 during transport. Additionally, depuration enables the efficient reduction of TVC and *E. coli*, but not *Vibrio* spp. This clearly demonstrates that *E. coli* is an insufficient microbiological indicator of the healthiness of mollusc. Optimal chilling temperatures should be used to transport/store both clam species to maintain high quality and to avoid *Vibrio* spp. growth. In such conditions, *R. philippinarum* and *V. pullastra* can be stored during more ten and two days, respectively compared to stressful temperatures (22 °C). Future studies are still required to assess the efficacy of the depuration process in reducing pathogenic *Vibrio* strains, such as *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* naturally accumulated in clams, as these strains are the major health concern in shellfish.

Acknowledgements

This study was supported by the SHELLPLANT research project (ref. 232273) funded by the European Commission (FP7). The first author acknowledges a PhD scholarship of the Portuguese Foundation for Science and Technology (FCT; Ref. SFRH/BD/69274/2010), whereas the last two authors were supported by a research contract from the Ciência 2008 Program of FCT.

References

Ali, F., Nakamura, K., 1999. Effect of temperature and relative humidity on the tolerance of the Japanese clam, *Ruditapes philippinarum* (Adams & Reeve), to air exposure. Aquaculture Research 30, 629–636.

Ali, F., Nakamura, K., 2000. Metabolic characteristics of the Japanese clam *Ruditapes philippinarum* (Adams & Reeve) during aerial exposure. Aquaculture Research 31, 157–165.

Ali, F., Nakamura, K., Yamasaki, S., 1999. Physiological characteristics of aerial survival in Japanese clam, *Ruditapes philippinarum* (Adams and Reeve). Suisanzoshoku 47 (3), 381–389.

Barile, N.B., Scopa, M., Nerone, E., Mascilongo, G., Recchi, S., Cappabianca, S., Antonetti, L., 2009. Study of the efficacy of closed cycle depuration system on bivalve molluscs. Veterinaria Italiana 45 (4), 555–566.

Bernárdez, M., Pastoriza, L., 2011. Quality of live packaged mussels during storage as a function of size and oxygen concentration. Food Control 22 (2), 257–265.

Berthou, P., Poutiers, J.M., Goulletquer, P., Dao, J.C., 2005. Shelled molluscs. In: Fisheries and Aquaculture. Encyclopedia of Life Support Systems (EOLSS), Developed under the Auspices of the UNESCO, vol. II. Eolss Publishers, Oxford, UK.

Brock, V., Kennedy, V., Brock, A., 1986. Temperature dependency of carbohydrase activity in the hepatopancreas of thirteen estuarine and coastal bivalve species from the North American east coast. Journal of Experimental Marine Biology and Ecology 103, 87–101.

Brogueira, M.J., Cabeçadas, G., 2006. Identification of similar environmental areas in Tagus estuary by using multivariate analysis. Ecological Indicators 6, 508–515.

Cao, R., Xue, C.H., Liu, Q., Xue, Y., 2009. Microbiological, chemical, and sensory assessment of Pacific Oysters (*Crassostrea gigas*) stored at different temperatures. Czech Journal of Food Sciences 27, 102–108.

Cheesborough, M., 1991. Medical laboratory manual for tropical countries. Microbiology, Tropical Health Technology 11, 265–270.

CIESM, 2012. CIESM Atlas of Exotic Species in the Mediterranean. http://www.ciesm.org/atlas/Ruditapesphilippinarum.html (accessed 24.09.12.).

Colwell, R.R., Liston, J., 1960. Microbiology of shellfish. Bacteriological study of the natural flora of Pacific oysters (*Crassostrea gigas*). Applied Microbiology 8, 104–109.

Cook, D.W., 1991. Indicators and alternate indicators of growing waters. In: Ward, D.R., Ackney, C.H. (Eds.), Microbiology of Marine Food Products AVI Book. Van Nostrand Reinhold, New York, US, pp. 19–39.

Cozzi, L., Suffredini, E., Ciccaglioni, G., Croci, L., 2009. Depuration treatment of mussels experimentally contaminated with *V. parahaemolyticus* and *V. vulnificus*. In: Proceeding ICMSS09, Nantes, France.

Croci, L., Suffredini, E., Cozzi, L., Toti, L., 2002. Effects of depuration of molluscs experimentally contaminated with *Escherichia coli*, *Vibrio cholerae* O1 and *Vibrio parahaemolyticus*. Journal of Applied Microbiology 92, 460–465.

De Zwaan, A., Babarro, J.M.F., Monari, M., Cattani, O., 2002. Anoxic survival potential of bivalves: (arte)facts. Comparative Biochemistry and Physiology Part A – Molecular & Integrative Physiology 131A (3), 615–624.

Delgado, M., Pérez-Camacho, A., 2007. Comparative study of gonadal development of *Ruditapes philippinarum* (Adams and Reeve) and *Ruditapes decussatus* (L.) (Mollusca: Bivalvia): influence of temperature. Scientia Marina 71, 471–484.

DePaola, A., Nordstrom, J.L., Bowers, J.C., Wells, J.G., Cook, D.W., 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Applied and Environmental Microbiology 69, 1521–1526.

Duan, J., Su, Y.-C., 2005. Occurrence of *Vibrio parahaemolyticus* in two Oregon oyster-growing bays. Journal of Food Science 70, 58–63.

EC, 2001. Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on *Vibrio vulnificus* and *Vibrio parahaemolyticus* (in Raw and Undercooked Seafood). European Commission Health & Consumer Protection Directorate General. Directorate C—Scientific Opinions, pp. 1–64.

EC, 2004a. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for food of animal origin. Official Journal of the European Union L226, 22–82.

EC, 2004b. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Official Journal of the European Union L226, 83–127.

Ekanem, E.O., Adegoke, G.O., 1995. Bacteriological study of West African clam (Egeria radiate Lamarch) and their overlying waters. Food Microbiology 12, 381–385.

El-Gamal, M.M., 2011. The effect of depuration on heavy metals, petroleum hydrocarbons, and microbial contamination levels in *Paphia undulata* (Bivalvia: Veneridae). Czech Journal of Animal Science 56 (8), 345–354.

El-Shenawy, N.S., 2004. Heavy metal and microbial depuration of the clam *Ruditapes decussatus* and its effect on bivalve behavior and physiology. Environmental Toxicology 19, 143–153.

Eyles, M.J., Davey, G.R., 1984. Microbiology of commercial depuration of the Sydney rock oyster, *Crassostrea commercialis*. Journal of Food Protection 47, 703–706.

FAO, 2012a. Fisheries and Aquaculture Information and Statistics Service. http://www.fao.org/fishery/statistics/en (accessed 24.09.12.).

FAO, 2012b. Species Fact Sheets: *Ruditapes philippinarum* (Adams & Reeve, 1850). http://www.fao.org/fishery/species/3543/en (accessed 24.09.12.).

Flassch, J.P., Leborgne, Y., 1992. Introduction in Europe, from 1972 to 1980, of the Japanese Manila clam (*Tapes philippinarum*) and the effects on aquaculture production and natural settlement. ICES Marine Science Symposium 194, 92–96.

Gaspar, M.B., 2010. Distribuição, abundância e estrutura demográfica da amêijoa-japonesa (*Ruditapes philippinarum*) no Rio Tejo, p. 6. Relatório do IPIMAR.

Gooch, J.A., DePaola, A., Bowers, J., Marshall, D.L., 2002. Growth and survival of *Vibrio parahaemolyticus* in postharvest American oysters. Journal of Food Protection 65, 970–974.

Gosling, E., 2002. Bivalve Molluscs: Biology, Ecology and Culture. Fishing New Books, Blackwell Publishing, Oxford, UK.

Howard, G., Heron, J., Turriff, J., Kinnear, S., 2003. Preliminary Trials to Assess the Viability of Purifying *Venerupis* spp. by Depuration Using Ultra Violet Sterilisation. Fisheries Research Services Internal Report No 09/03. Marine Laboratory, Aberdeen, UK, p. 16.

ICMSF, 1986. Microorganisms in Foods 2. Sampling for Microbiological Analysis: Principles and Specific Applications, second ed.. In: International Commission on Microbiological Specifications for Foods University of Toronto Press/Blackwell Scientific Publications, Toronto, p. 131.

ISO, 2001. ISO 16649-2:2001. Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of β-glucuronidase-positive *Escherichia coli* – Part 2: Colony-count Technique at 44 °C Using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. International Organisation for Standardisation, Geneve, Switzerland.

Jensen, A.C., Humphreys, J., Caldow, R.W.G., Grisley, C., Dyrynda, P.E.J., 2004. Naturalization of the Manila clam (*Ruditapes philippinarum*), an alien species, and establishment of a clam fishery within Poole Harbour, Dorset. Journal of the Marine Biological Association of the United Kingdom 84, 1069–1073.

Jones, G.G., Sanford, C.L., Jones, B.L., 1993. Manila Clams: Hatchery and Nursery Methods. Innovative Aquaculture Products Ltd, p. 70.

Kaneko, T., Colwell, R.R., 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. Journal of Bacteriology 113, 24–32.

Lees, D., Younger, A., Dore, B., 2010. Depuration and relaying. In: Rees, G., Pond, K., Kay, D., Bartram, J., Santo Domingo, J. (Eds.), Safe Management of Shellfish and Harvest Waters. World Health Organization (WHO), IWA Publishing, London, UK, p. 37.

Lemarchand, K., Masson, L., Brousseau, R., 2004. Molecular biology and DNA microarray technology for microbial qualify monitoring of water. Critical Reviews Microbiology 30 (3), 145–172.

Loosanoff, V., 1953. Behavior of oysters in water of low salinities. Proceedings of the National Shellfisheries Association 43, 131–151.

Lopez-Joven, C., Ruiz-Zarzuela, I., de Blas, I., Furones, M.D., Roque, A., 2011. Persistence of sucrose fermenting and nonfermenting vibrios in tissues of Manila clam species, *Ruditapes philippinarum*, depurated in seawater at two different temperatures. Food Microbiology 28, 951–956.

Macedo, M.C.C., Macedo, M.I.C., Borges, J.P., 1999. Conchas Marinhas de Portugal. Editorial Verbo, Lisbon.

Maffei, M., Vernocchi, P., Lanciotti, R., Guerzoni, M.E., Belletti, N., Gardini, F., 2009. Depuration of striped Venus clam (*Chamelea gallina* L.): effects on microorganisms, sand content, and mortality. Journal of Food Science 74 (1), M1–M7.

Marino, A., Lombardo, L., Fiorentino, C., Orlandella, B., Monticelli, L., Nostro, A., Alonzo, V., 2005. Uptake of *Escherichia coli*, *Vibrio cholera* non-O1 and *Enterococcus durans* by, and depuration of mussels (*Mytilus galloprovincialis*). International Journal of Food Microbiology 99, 281–286.

Martínez, O., Rodríguez-Calleja, J.M., Santos, J.A., Otero, A., García-López, M.L., 2009. Foodborne and indicator bacteria in farmed molluscan shellfish before and after depuration. Journal of Food Protection 72, 1443–1449.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States. Emerging Infectious Diseases 5, 607–625.

Ripabelli, G., Sammarco, M.L., Grasso, G.M., Fanelli, I., Caprioli, A., Luzzi, I., 1999. Occurrence of *Vibrio* and other pathogenic bacteria in *Mytilus galloprovincialis* (mussels) harvested from Adriatic Sea, Italy. International Journal of Food Microbiology 49 (1–2), 43–48.

Sea Grant, 2012. Managing the Cold Chain for Quality and Safety. http://seafood.ucdavis.edu/pubs/coldchain.doc (accessed 24.09.12.).

Sobral, P., Widdows, J., 1997. Effects of elevated temperatures on the scope for growth and resistance to air exposure of the clam *Ruditapes decussatus* (L.), from southern Portugal. Scientia Marina 61, 163–171.

Su, Y.-C., Liu, C., 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. Food Microbiology 24, 549–558.

Timoney, J.F., Abston, A., 1984. Accumulation and elimination of *Escherichia coli* and *Salmonella typhimurium* by hard clams in an in vitro system. Applied and Environmental Microbiology 47 (5), 986–988.

Uzaki, N., Kai, M., Aoyama, H., Suzuki, T., 2003. Changes in mortality rate and glycogen content of the Manila clam *Ruditapes philippinarum* during the development of oxygen-deficient waters. Fisheries Science 69, 936–943.

Vasconcelos, G.J., Lee, J.S., 1972. Microbial flora of Pacific oysters (Crassostrea gigas) subjected to ultraviolet-irradiated seawater. Applied Microbiology 23, 11–16.

Wittman, R.J., Flick, G.J., 1995. Microbial contamination of shellfish: prevalence, risk to human health, and control strategies. Annual Review of Public Health 16, 123–140.

CHAPTER 5

Physiological responses to depuration and transport of native and exotic clams at different temperatures

Anacleto P., Maulvault A. L., Barrento S., Mendes R., Nunes M. L., Rosa R. and Marques A.

(2013)

Aquaculture, 408–409: 136–146

DOI: 10.1016/j.aquaculture.2013.05.035

Aquaculture 408–409 (2013) 136–146

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Physiological responses to depuration and transport of native and exotic clams at different temperatures

Patrícia Anacleto [a], Ana Luísa Maulvault [a], Sara Barrento [b], Rogério Mendes [a], Maria Leonor Nunes [a], Rui Rosa [c], António Marques [a,*]

[a] Portuguese Institute of Sea and Atmosphere (IPMA, I.P.), Av. Brasília, 1449-006 Lisbon, Portugal
[b] Centre for Sustainable Aquaculture Research, Department of Biosciences, College of Science, Swansea University, Singleton Park, Swansea, SA2 8PP, UK
[c] Guia Marine Laboratory, Center of Oceanography, Faculty of Sciences, University of Lisbon (FCUL), Campo Grande, 1749-016 Lisboa, Portugal

ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form 14 May 2013
Accepted 30 May 2013
Available online 7 June 2013

Keywords:
Clams
Depuration
Transport
LT50
Survival
Physiology

ABSTRACT

The trade of live bivalves is a complex chain (harvesting, depuration, transportation), where animals are affected by several stressors that reduce animal condition and promote mortalities, with the consequent economic losses. The aim of this study was to investigate the physiological responses (survival rates, condition index, breakdown products of ATP and glycogen content) of two bivalve species from the Tagus estuary, *Venerupis pullastra* (native clam) and *Ruditapes philippinarum* (exotic clam) during two days of depuration and subsequent transport in semi-dry conditions at two temperatures (4 and 22 °C) until reaching 50% lethal time (LT50). Depuration did not negatively affect both species, even enabling additional two day survival extension until attaining LT50. In contrast, transport was an important stressor, with temperature contributing greatly for mortality: *R. philippinarum* showed higher survival rates than *V. pullastra*, always reaching LT50 later, especially at 4 °C. The exotic clam survived nine days more at 4 °C, but only one day more at 22 °C than the native species. Nevertheless, native clams showed higher condition index and glycogen content, and also lower nucleotide K-value and adenylate energy charge than exotic clams. The best semi-dry transport conditions to maintain good physiological conditions and high quality of clams should be performed at low temperatures (4 °C).

1. Introduction

Clams represent around 26% of mollusc production in Portugal (5276 tonnes; FAO, 2012a), and Tagus estuary is one of the key production and harvesting sites. Pullet carpet shell clam (*Venerupis pullastra*) and Manila clam (*Ruditapes philippinarum*) are the most successfully bivalve molluscs commercialized for human consumption in this estuary. *V. pullastra* is a native species of Tagus estuary that lives in a shallow burrow just under the surface in sand, mud or gravel, and usually occurs in the intertidal zone down to about 40 m (FAO, 2012b). For this species, the optimal growth temperature is around 20 °C and the optimal salinity is between 30 and 40. In contrast, *R. philippinarum* is an allochthonous species introduced in the beginning of the 1970s for culture purposes in North European Atlantic and Mediterranean coastal waters (Flassch and Leborgne, 1992). This species buries few centimeters into the sediment and usually occurs in the lower region of estuaries (Lee, 1996). Their optimal growth temperature ranges from 20 °C to 24 °C (Solidoro et al., 2000) and supports salinities between 16 and 36, but its optimum salinity is between 20 and 26 (Nie, 1991). The relevance of this exotic species in Portugal, particularly in Tagus estuary, has rapidly increased in the last decade, mostly due to their better tolerance to variations of temperature and salinity, high growth rate, great capacity to adapt to new environments, dispersion facility and capacity to live at high animal densities (Gaspar, 2010).

Nowadays, the trade chain of bivalves is complex, with animals being subjected to several stressors during capture, depuration, transport and storage, including prolonged periods of air exposure and temperature fluctuation (Lee et al., 2008). The stressors strongly decrease animal quality, cause important changes in the animal metabolism and contribute to significant economical losses along the trade chain. Therefore, monitoring bivalves' physiological conditions is essential to avoid mortalities and optimize stakeholders profitability, thus ensuring minimal quality losses to consumers and, consequently, improve the trade chain. Beyond physiological tests, also biochemical and behavioral tests can give a more complete picture of organisms' reaction to stress (Maguire et al., 1999). Others tests include non-invasive (e.g. survival rate, condition index) and invasive processes (e.g. different breakdown products of adenosine triphosphate — ATP, glycogen content).

Depuration is a process required for bivalves used for human consumption, which consists in maintaining bivalves up to 48 h in sterile seawater with sufficient oxygen and without any feed to reduce the content of potential pathogenic microorganisms, particularly *Escherichia coli*,

* Corresponding author. Tel.: +351 213 027 025; fax: +351 21 3015948.
E-mail address: amarques@ipma.pt (A. Marques).

0044-8486/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquaculture.2013.05.035

to ensure healthy and safety products. However, in Mediterranean countries several fishermen trade non-depurated bivalves without sanitary control, particularly *R. philippinarum*. Additionally, bivalves are often subjected to wide temperature variations and not maintained in refrigerated conditions along the trade chain. On the other hand, the effect of depuration in physiological status of the organisms during and after this period is relatively unknown. Several studies have been carried out to evaluate the physiological effect of temperature during aerial exposure of *R. philippinarum* (Ali and Nakamura, 1999, 2000; Ali et al., 1999; Uzaki et al., 2003), as well as the effect of depuration in chemical contamination (Freitas et al., 2012), but no studies were performed with *V. pullastra*.

In this context, the aim of this study was to compare the physiological responses (survival profile, condition index, breakdown products of ATP and glycogen content) of two clam species, the native *V. pullastra* and the exotic *R. philippinarum* from Tagus estuary in two critical steps of their trade chain, i.e. depuration (two days) and subsequent transport in semi-dry conditions at two temperatures (4 and 22 °C) until reaching 50% lethal time (LT50).

2. Materials and methods

2.1. Collection of samples and treatments

Two clam species were harvested using bottom trawls in Summer 2011 from two fishing areas: the pullet carpet shell clam (*V. pullastra*) was harvested from Trafaria, located in the mouth of Tagus estuary during September 2011, and the Manila clam (*R. philippinarum*) was harvested from Alcochete, located in the upstream part of the estuary during October 2011 (Fig. 1). Since the species have different reproductive cycles, the sampling occurred at two different months to guarantee the same conditions of their annual cycle, i.e. during their reproductive stage before spawning. Tagus estuary is one of the largest estuaries of the European west coast located in the most populated area of Portugal, the capital, Lisbon, with a broad shallow bay covering an area of about 320 km^2 (Brogueira and Cabeçadas, 2006). After harvest, both clams were immediately stored at 4 °C and transported in thermal insulated boxes until their arrival to the laboratory (approximately a two hour transport).

Specimen biometric data was obtained (mean ± standard deviation; n = 100) for *V. pullastra* (weight: 9.5 ± 3.0 g; shell length: 35.4 ± 4.1 mm; shell width: 17.1 ± 2.1 mm; shell height: 23.4 ± 2.9 mm) and *R. philippinarum* (weight: 17.6 ± 5.7 g; shell length: 40.5 ± 4.2 mm; shell width: 21.5 ± 2.8 mm; and shell height: 29.7 ± 3.2 mm). In total, 2264 animals were sampled (1180 native clams and 1084 exotic clams). Fig. 2 shows the schematic representation of the experimental design used. Briefly, each species was divided in two batches: in one batch the clams were depurated during two days followed by simulated transport in semi-dry conditions at either 4 °C (treatment DT4) or at 22 °C (treatment DT22). The transport was simulated until 50% of all clams were dead, that is the 50% lethal time (LT50). The other batch of clams was not depurated it was only tested for simulated transport in semi-dry conditions as described before. Temperature controlled chambers set at 4 or 22 °C were used to simulate transport, where clams packed in 1 kg net bags were placed inside plastic boxes with draining holes and covered with wet cloth. Thermometers and data-loggers were used to monitor temperature levels inside the chambers. Since clams are sensitive to physical damage, the animals were carefully handled to minimize mortalities. Daily, mortality was registered in each treatment and the behavioral activity was checked in one hundred clams per treatment using the criteria described by El-Shenawy (2004) with some modifications (Table 1) until reaching LT50. Per treatment and sampling day, 25 specimens were randomly collected and measured for assessment of condition index (only at transport days 0, 1, 2, 3 and LT50), whereas for biochemical/physiological analyses, the whole edible tissue and adductor muscle of 10 clams were vacuum-packed and immediately frozen at −80 °C for nucleotides and glycogen analyses (every day until transport day 5 and then two in two days until reaching LT50). For glycogen analysis, the samples were then freeze-dried during 48 h and the dry samples were powdered and stored at −80 °C until analyses. All analyses were carried out in triplicate.

2.1.1. Depuration experiment

Depuration started 2 h after harvesting and was carried out in two recirculated tanks (length, 31 cm; height, 40 cm) containing 15 L of UV sterilized seawater. The inlet flow rate was 87 L h^{-1} kg^{-1}. Each tank had 2 kg of clams, corresponding to a clam to water ratio similar to those usually employed at Portuguese depuration facilities. Clams were not fed during the experimental period and seawater was kept with continuous aeration (9.56 mg L^{-1}) and constant temperature (18.3 °C), salinity (35.5 g L^{-1}) and pH (6.98). Bivalve samples were

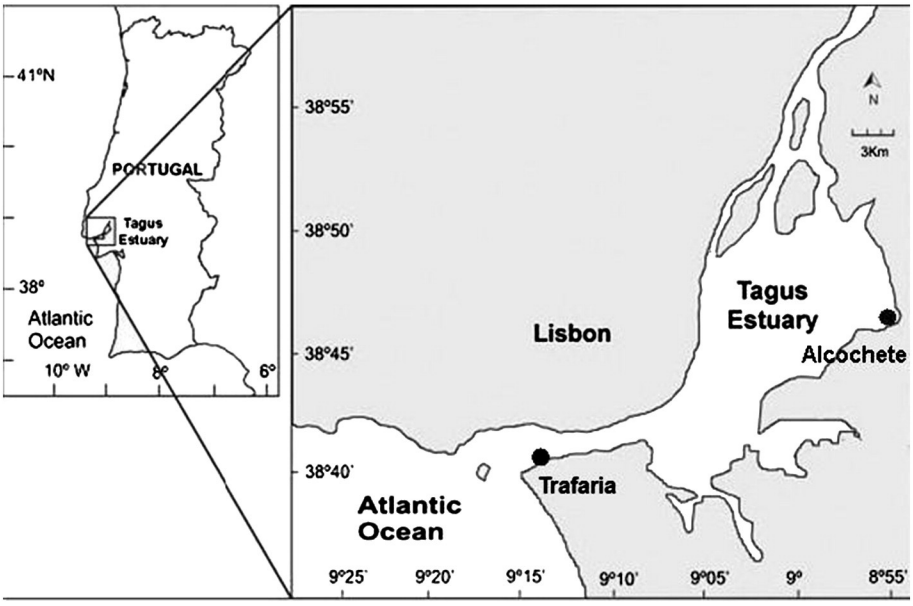


Fig. 1. Map of Portugal with enlarged portion of the Tagus estuary. Dark dots indicate the samplings sites (Trafaria for *V. pullastra* and Alcochete for *R. philippinarum*).

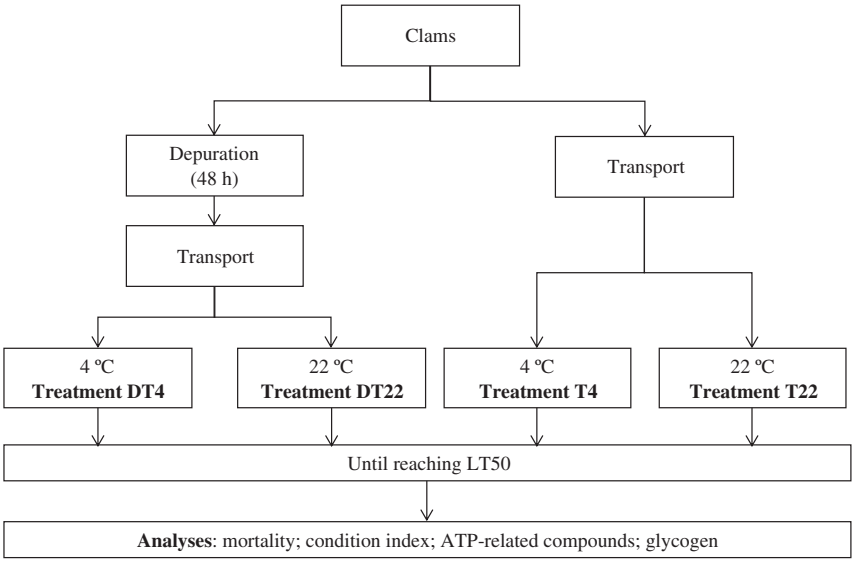


Fig. 2. Schematic representation of the experimental design.

collected at 0, 1 and 2 days for further biochemical/physiological analyses.

2.2. Physiological analyses

2.2.1. Condition index

The condition index (CI) was calculated according to the following equation in wet tissue (Maguire et al., 1999):

$$CI = \frac{[\text{weight}]}{[\text{shell length}] \times [\text{shell height}] \times [\text{shell width}]} \times 10,000$$

2.2.2. Nucleotides

The extraction procedure was based on the method of Ryder (1985). Briefly, the edible tissue of one clam was extracted with of 0.6 M perchloric acid (1:5 w/v; Panreac, Barcelona, Spain) at 0 °C for 1 min using an Ultra-Turrax homogenizer (T25 basic IKA-WERKE, Staufen, Germany). This process was done on three separate clams (i.e. in triplicate). The homogenate was centrifuged at 20,000 *g* during 10 min at 0 °C, and 10 mL of supernatant was neutralized to pH 6.90 with 1 M potassium hydroxide solution (Panreac, Barcelona, Spain). The supernatant was maintained at 0 °C during 30 min to precipitate potassium perchlorate that was removed by vacuum filtration with sintered glass. The filtrate was diluted to the final volume of 20 mL with Milli-Q purified water, previously filtered (0.20 μm pore size) and stored at −80 °C in vials until high performance liquid chromatography (HPLC) analysis.

HPLC nucleotide analysis was carried out in a Hewlett Packard 1050 HPLC system (Hewlett Packard GmbH, Waldbronn, Germany) at a fixed wavelength of 254 nm. Aliquots of sample extracts (20 μL) were injected in duplicate. Separation of nucleotides was achieved by a Hewlett Packard LiChrosorb RP-18 column (10 μm, 250 × 4.6 mm; VDS optilab, Berlin, Germany) maintained at 30 °C. The mobile phase was 0.04 M potassium dihydrogen orthophosphate (Riedel de Haën, Selze, Germany) and 0.06 M dipotassium hydrogen orthophosphate (Fluka, Berlin, Germany) dissolved in Milli-Q purified water (pH 6.90), at a flow rate of 1.6 mL min^{-1} for 23 min. The peaks obtained from extracts were identified by comparison with the retention time of Sigma-Aldrich (USA) standard solutions. All solutions were filtered (0.45 μm pore size) prior to injection.

Nucleotides comprised adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx). Results were expressed as micromoles of nucleotide per gram of clam edible tissue (μmol g^{-1} wet weight).

The K-value (Saito et al., 1959) and adenylate energy charge (A.E.C. value; Atkinson, 1968) were calculated based on the following equations:

$$K(\%) = \left[\frac{(Hx + HxR)}{(ATP + ADP + AMP + IMP + Hx + HxR)}\right] \times 100$$

$$A.E.C.(\%) = \left[\frac{(ATP + 0.5ADP)}{(ATP + ADP + AMP)}\right] \times 100.$$

2.2.3. Glycogen

Glycogen concentrations in bivalve meat samples were determined according to the method described by Viles and Silverman (1949). Briefly, adductor muscle samples (25 mg dry weight) were hydrolyzed with 15 mL of 33% potassium hydroxide (Panreac, Barcelona, Spain), in a water bath at 100 °C during 15 min. The absorbance was measured at 620 nm (spectrophotometer UNICAM UV–Vis, Helios, United Kingdom). A calibration curve was prepared using glycogen (Sigma, USA) as standard. The anthrone-reagent was prepared with 38 mL concentrated sulphuric acid (Fluka, Riedel-de Haën, Germany), 15 mL distilled water and 0.075 g anthrone. The results were expressed as g 100 g^{-1} wet weight. This process was done separately from three individual clams (i.e. in triplicate).

2.3. Statistical analysis

The statistical analysis was performed using the software STATISTICA™ 7.0 (Statsoft, Inc., Tulsa, OK, USA). Two-way analysis

Table 1
Summary of criteria used for scoring behavioral clam activity.

Score	Criterion
0	Shell valve closed
1	Shell valve merely opened but mantle edge not clearly visible
2	Shell valve opened and mantle edge clearly visible
3	Shell valve opened and siphons extending up to half their full length
4	Shell valve opened and both siphons and foot protruding (i.e. dead clam)

of variance (ANOVA) was conducted to detect significant differences in condition index, ATP and related-compounds and glycogen content between clam species and transport temperature (4 and 22 °C). Additionally, one-way ANOVA was used to evaluate the effect of depuration and transport time in each treatment. Repeated measures ANOVA was used to determine the influence of depuration and transport temperatures on clam condition index. Whenever necessary, data was transformed to satisfy normal distribution and homoscedasticity requirements. If transformed data could not meet these assumptions, non-parametric analysis of variance (Kruskal–Wallis) was performed, followed by non-parametric multiple comparison test (Dunn test). Correlations between the studied physiological parameters were analyzed for each treatment with Pearson's correlation coefficients for linear correlation. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Survival

Fig. 3 shows the changes in survival rate of native and exotic clams during depuration and transport, or only during transport without depuration. During depuration, no significant differences were found between clam species (varied only between 97 and 100%). A significant difference in survival of both species was observed during transport of depurated ($F = 5.2$, $p < 0.05$) and non-depurated clams ($F = 5.0$, $p < 0.05$) (Table 2). *R. philippinarum* always had a higher survival rate than *V. pullastra*. These differences were found after two days of transport at both temperatures in depurated clam species, while for non-depurated clams such differences were observed after two and four days of transport at 22 and 4 °C, respectively (Fig. 3).

Moreover, the decrease in survival was always faster in both clams exposed to higher temperatures (22 °C) reaching LT50 more rapidly (three days for *V. pullastra* and four days for *R. philippinarum*; Fig. 3) than at lower temperatures (4 °C; five days for *V. pullastra* and fourteen days for *R. philippinarum*). A significant influence of transport temperature was found in survival of depurated ($F = 5.2$, $p < 0.05$) and non-depurated clams ($F = 4.5$, $p < 0.05$) (Table 2). These differences were only observed after three days of transport in both species subjected to depuration (Fig. 3A), while for non-depurated clams were found after two days of transport in *V. pullastra* and four days of transport in *R. philippinarum* (Fig. 3B).

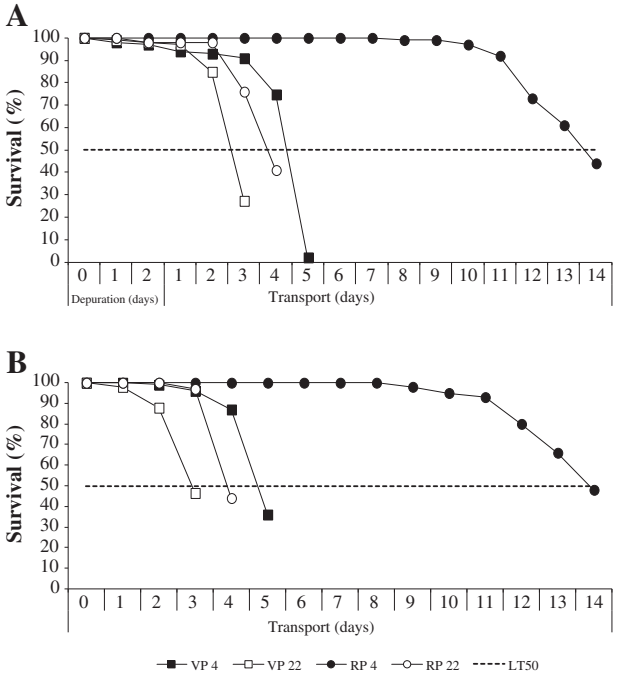


Fig. 3. Survival rate (%) of native (*Venerupis pullastra*, VP) and exotic (*Ruditapes philippinarum*, RP) clams subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C. The dashed line indicates the LT50 values.

Table 2
Results of two-way ANOVA evaluating the effects of transport temperature (4 and 22 °C) and species (*Venerupis pullastra* and *Ruditapes philippinarum*) on survival, CI, K and A.E.C. values and glycogen content.

Parameters	Depurated				Non-depurated			
	df	MS	F	p	df	MS	F	p
Survival								
Temperature (T)	1	1 348.5	5.2	0.034*	1	666.1	4.5	0.047*
Species (S)	1	1 356.6	5.2	0.034*	1	751.0	5.0	0.036*
T × S	1	328.9	1.3	0.274	1	543.9	3.6	0.070
Error	20	260.7			20	148.9		
CI								
Temperature (T)	1	0.134	36.0	0.000*	1	0.005	2.9	0.092
Species (S)	1	0.010	2.8	0.097	1	0.023	14.0	0.000*
T × S	1	0.019	5.1	0.025*	1	0.001	0.71	0.399
Error	260	0.004			260	0.002		
K-value								
Temperature (T)	1	198.2	4.7	0.034*	1	849.5	26.2	0.000*
Species (S)	1	3.2	0.077	0.782	1	28.3	0.871	0.355
T × S	1	6.6	0.157	0.693	1	68.3	2.1	0.153
Error	52	42.0			52	32.5		
A.E.C. value								
Temperature (T)	1	771.8	6.2	0.016*	1	5.0	0.144	0.706
Species (S)	1	10.7	0.085	0.771	1	497.8	14.2	0.000*
T × S	1	3 090.5	24.7	0.000*	1	18.1	0.519	0.474
Error	52	125.3			52	34.9		
Glycogen								
Temperature (T)	1	2.8	45.7	0.000*	1	0.155	1.4	0.246
Species (S)	1	16.3	260.0	0.000*	1	11.9	107.1	0.000*
T × S	1	0.028	0.440	0.512	1	0.008	0.070	0.793
Error	32	0.062			32	0.111		

Abbreviations: CI, condition index. Asterisk indicates significant differences.

R. philippinarum revealed statistically positively correlations between survival and ATP (depurated clams) and CI (all treatments, except DT4), whereas significant negative linear relationships were found between survival and ADP (T4), HxR (non-depurated clams), Hx (all treatments, except T4), K-value (in depurated clams), A.E.C. (all treatments, except T4) and glycogen (T4) (Table 3). In contrast, for *V. pullastra* a significant positive correlation was found between survival and CI (depurated clams), ADP (DT22) and glycogen (clams transported at 22 °C) (Table 4). Significant negative linear relationships were found between survival and ATP (T22), IMP (clams transported at 22 °C) and HxR, Hx, and K-value (all treatments) (Table 4).

3.2. Physiological analyses

3.2.1. Condition index

In the beginning of the experiment, CI was significantly higher in depurated *R. philippinarum* than *V. pullastra* (Fig. 4A). Yet, a significant influence of species in CI was only observed in non-depurated clams ($F = 14.0$, $p < 0.05$; Table 2), where *V. pullastra* showed significantly higher CI than *R. philippinarum* after 3 days of transport at 4 °C (Fig. 4B). During depuration CI did not show significant differences in both clam species (Fig. 4A). However, throughout the transport period CI gradually decreased in all treatments of depurated and non-depurated clams, being immediately significant after one day transport at both temperatures and reaching a minimum CI at LT50, except at 4 °C for non-depurated *V. pullastra* (decreased only after 3 days) (Fig. 4).

Concerning transport temperatures, a significant influence of temperature was only found in depurated clams ($F = 36.0$, $p < 0.05$;

Table 3
Pearson correlation coefficients between survival, CI, ATP-related compounds (IMP, ATP, ADP, AMP, Hx and HxR), K and A.E.C. values and glycogen content in each treatment of *Ruditapes philippinarum*.

		Survival	CI	ATP	ADP	AMP	IMP	HxR	Hx	K-value	A.E.C. value	Glycogen
DT4	Survival	1.00										
	CI	0.44	1.00									
	ATP	0.69*	0.25	1.00								
	ADP	0.40	−0.08	−0.11	1.00							
	AMP	−0.04	0.10	0.14	−0.69*	1.00						
	IMP	0.30	0.19	0.27	−0.11	0.40	1.00					
	HxR	0.48	0.26	0.50	−0.25	0.60*	−0.04	1.00				
	Hx	−0.71*	−0.36	−0.82*	0.06	−0.31	−0.69*	−0.48	1.00			
	K-value	−0.78*	−0.37	−0.72*	−0.06	−0.32	−0.78*	−0.39	0.95*	1.00		
	A.E.C. value	−0.86*	−0.37	−0.75*	−0.22	−0.18	−0.65*	−0.45	0.94*	0.97*	1.00	
	Glycogen	−0.20	−0.05	−0.12	−0.30	0.46	0.84*	−0.33	−0.25	−0.37	−0.16	1.00
DT22	Survival	1.00										
	CI	0.58*	1.00									
	ATP	0.58*	0.54*	1.00								
	ADP	−0.01	0.14	−0.04	1.00							
	AMP	0.03	0.12	0.28	−0.44	1.00						
	IMP	0.26	0.24	0.48	−0.38	0.84*	1.00					
	HxR	0.41	0.10	0.25	−0.25	−0.05	0.46	1.00				
	Hx	−0.88*	−0.45	−0.56*	0.37	−0.06	−0.37	−0.61*	1.00			
	K-value	−0.65*	−0.29	−0.49	0.58*	−0.30	−0.65*	−0.81*	0.88*	1.00		
	A.E.C. value	−0.79*	−0.36	−0.51	0.48	−0.13	−0.48	−0.77*	0.96*	0.97*	1.00	
	Glycogen	−0.22	0.02	0.14	−0.35	0.95*	0.72*	−0.25	0.18	−0.06	0.13	1.00
T4	Survival	1.00										
	CI	0.82*	1.00									
	ATP	0.44	0.86*	1.00								
	ADP	−0.78*	−0.37	0.12	1.00							
	AMP	−0.25	−0.12	0.12	0.62	1.00						
	IMP	−0.45	−0.48	−0.28	0.57	0.90*	1.00					
	HxR	−0.79*	0.32	−0.18	−0.91*	−0.26	−0.21	1.00				
	Hx	−0.65	−0.86*	−0.79*	0.23	0.18	0.43	−0.17	1.00			
	K-value	−0.34	0.20	0.57	0.61	−0.06	−0.24	−0.81*	−0.39	1.00		
	A.E.C. value	−0.61	−0.49	−0.31	0.23	−0.49	−0.37	−0.54	0.48	0.38	1.00	
	Glycogen	−0.91*	−0.92*	−0.66	0.62	0.28	0.60	−0.53	0.68*	0.06	0.39	1.00
T22	Survival	1.00										
	CI	0.83*	1.00									
	ATP	0.43	0.70*	1.00								
	ADP	−0.03	0.06	−0.26	1.00							
	AMP	−0.12	−0.45	−0.65	0.00	1.00						
	IMP	0.20	−0.19	−0.63	0.17	0.92*	1.00					
	HxR	−0.71*	0.55	0.35	−0.46	−0.42	−0.18	1.00				
	Hx	−0.79*	−0.75*	−0.53	−0.11	−0.09	−0.27	−0.20	1.00			
	K-value	−0.57	−0.25	0.06	0.16	−0.70*	−0.79*	−0.12	0.70*	1.00		
	A.E.C. value	−0.81*	−0.88*	−0.66	−0.09	0.67*	0.36	−0.73*	0.54	0.00	1.00	
	Glycogen	−0.08	−0.31	−0.69*	0.58	0.81*	0.86*	−0.56	−0.14	−0.49	0.46	1.00

Abbreviations: TVC, total viable counts, DT4 and DT22, depurated and transported clams at 4 or 22 °C, T4 and T22, non-depurated clams transported at 4 or 22 °C. Asterisk indicates significant differences.

during all transport in *R. philippinarum* and only at three days transport in *V. pullastra*) and also a temperature-species interaction ($F = 5.1$, $p < 0.05$) (Table 2; Fig. 4).

Concerning correlations, apart from those described previously, *R. philippinarum* CI showed a significant positive linear relationship with ATP in all treatments, except DT4, as well as negative linear relationship with Hx (non-depurated clams), A.E.C. (T22) and glycogen (T4) (Table 3). In contrast, statistically positive linear correlation was found between *V. pullastra* CI and ADP (clams transported at 4 °C), AMP (depurated clams), IMP (DT22) and glycogen (DT4) (Table 4). Additionally, statistically negative correlation was found with HxR, K and A.E.C. values (depurated clams) and with Hx (DT4) (Table 4).

3.2.2. Nucleotides

The pattern of ATP content and related compounds in depurated and non-depurated *R. philippinarum* and *V. pullastra* transported at 4 or 22 °C is shown in Figs. 5 and 6. In the beginning of the experiment, all nucleotides except HxR were significantly higher in *V. pullastra* than in *R. philippinarum*. Furthermore, very low concentrations (<0.10 μmol g^{-1}) of ATP and Hx were detected in both clam species and HxR only in native clams, whereas IMP and AMP were the most prominent nucleotides in both species (>0.65 μmol g^{-1}).

During the semi-dry transport period, AMP and IMP were present at higher concentrations in both clam species (Figs. 5 and 6). Moreover, the levels of HxR and Hx significantly increased during transport in all treatments of both clam species, except non-depurated exotic clams transported at 4 °C, as well as the degradation of AMP in several treatments. In native clams, ATP and ADP levels only significantly decreased in depurated specimens transported at 22 °C, whereas AMP increased (Fig. 5). In contrast, only depurated exotic clams at 4 °C revealed significantly ADP reduction throughout semi-dry transport, whereas ATP, HxR and Hx increased. Furthermore, AMP was reduced during transport mainly in non-depurated exotic clams, but IMP and Hx increased (Fig. 6).

A significant influence of species was observed for all nucleotides, except in ATP of non-depurated clams (Table 5). During the depuration period, only the levels of ATP and ADP significantly increased in native clams (Fig. 5). In contrast, in exotic clams the main changes were the significant decrease of ATP and HxR, and the statistical increase of ADP. Interestingly, AMP, IMP, HxR and Hx levels were significantly higher in all treatments of native clams compared to exotic clams at LT50 (Figs. 5 and 6).

Regarding transport temperature, a significant influence was observed in depurated (ATP, AMP, HxR and Hx) and non-depurated

Table 4
Pearson correlation coefficients between survival, CI, ATP-related compounds (IMP, ATP, ADP, AMP, Hx and HxR), K and A.E.C. values and glycogen content in each treatment of *Venerupis pullastra*.

		Survival	CI	ATP	ADP	AMP	IMP	HxR	Hx	K-value	A.E.C. value	Glycogen
DT4	Survival	1.00										
	CI	0.67*	1.00									
	ATP	0.22	−0.27	1.00								
	ADP	0.34	0.60*	0.82*	1.00							
	AMP	−0.17	0.70*	−0.58*	−0.81*	1.00						
	IMP	−0.15	0.00	0.23	0.16	0.35	1.00					
	HxR	−0.98*	−0.76*	−0.33	−0.47	0.33	0.19	1.00				
	Hx	−0.99*	−0.71*	−0.32	−0.47	0.31	0.18	0.99*	1.00			
	K-value	−0.97*	−0.78*	−0.38	−0.52*	0.34	0.12	1.00*	0.99*	1.00		
	A.E.C. value	0.29	−0.80*	0.85*	0.99*	−0.82*	0.17	−0.42	−0.42	−0.48	1.00	
	Glycogen	0.51	0.70*	−0.04	−0.12	0.21	−0.26	−0.41	−0.46	−0.37	−0.17	1.00
DT22	Survival	1.00										
	CI	0.85*	1.00									
	ATP	0.40	−0.52	1.00								
	ADP	0.70*	0.24	0.53	1.00							
	AMP	−0.31	0.63*	−0.62*	−0.40	1.00						
	IMP	−0.81*	0.66*	−0.50	−0.37	0.38	1.00					
	HxR	−0.88*	−0.82*	−0.46	−0.65*	0.58*	0.64*	1.00				
	Hx	−0.99*	−0.33	−0.36	−0.72*	0.22	0.80*	0.82*	1.00			
	K-value	−0.96*	−0.90*	−0.45	−0.73*	0.47	0.71*	0.98*	0.92*	1.00		
	A.E.C. value	0.44	−0.91*	0.83*	0.78*	−0.81*	−0.33	−0.61*	−0.39	−0.57*	1.00	
	Glycogen	0.82*	0.42	0.36	0.51	−0.24	−0.66*	−0.80*	−0.82*	−0.84*	0.33	1.00
T4	Survival	1.00										
	CI	0.41	1.00									
	ATP	0.30	−0.19	1.00								
	ADP	0.50	0.67*	0.62	1.00							
	AMP	0.03	0.47	0.86*	0.64	1.00						
	IMP	−0.64	0.02	−0.22	−0.14	0.28	1.00					
	HxR	−0.96*	−0.34	−0.47	−0.64	−0.17	0.71*	1.00				
	Hx	−0.75*	0.59	−0.39	−0.23	0.10	0.95*	0.80*	1.00			
	K-value	−0.99*	−0.49	−0.26	−0.50	0.04	0.70*	0.97*	0.79*	1.00		
	A.E.C. value	0.36	−0.37	0.91*	0.59	0.62	−0.54	−0.57	−0.63	−0.37	1.00	
	Glycogen	0.38	0.58	0.25	0.17	−0.19	−0.90*	−0.52	−0.82*	−0.46	0.60	1.00
T22	Survival	1.00										
	CI	−0.01	1.00									
	ATP	−0.91*	−0.37	1.00								
	ADP	0.70	0.17	−0.44	1.00							
	AMP	0.46	0.41	−0.77*	0.00	1.00						
	IMP	−0.76*	0.11	0.46	−0.98*	0.05	1.00					
	HxR	−0.98*	−0.09	0.90*	−0.78*	−0.51	0.80*	1.00				
	Hx	−0.99*	−0.28	0.88*	−0.79*	−0.43	0.82*	0.99*	1.00			
	K-value	−0.97*	−0.07	0.83*	−0.85*	−0.36	0.87*	0.99*	1.00*	1.00		
	A.E.C. value	−0.01	−0.13	0.37	0.67	−0.66	−0.64	−0.07	−0.10	−0.20	1.00	
	Glycogen	0.91*	0.54	−0.71	0.81*	0.13	−0.87*	−0.89*	−0.93*	−0.95*	0.28	1.00

Abbreviations: TVC, total viable counts, DT4 and DT22, depurated and transported clams at 4 or 22 °C, T4 and T22, non-depurated clams transported at 4 or 22 °C. Asterisk indicates significant differences.

clams (ADP, AMP, HxR and Hx) (Table 5). Figs. 7 and 8 reveal an overall increase in K-value and A.E.C. value in both clam species (except for A.E.C. value in non-depurated *V. pullastra* and transported at 4 °C). Initially, *R. philippinarum* showed significantly higher K-value (9%) than *V. pullastra* (3%), but lower A.E.C. value (12 and 14%, respectively). Overall, the K-value significantly increased in both clam species during transport and was always faster in specimens transported at higher temperatures (22 °C), attaining higher levels in exotic clams (30–40%), mainly in depurated specimens, compared to native clams (23–30%) at LT50. In contrast, A.E.C. value revealed a great oscillation in almost all treatments, but usually depurated clams as well as specimens transported at 22 °C showed higher levels. A significant influence of species was only observed for A.E.C. value in non-depurated clams ($F = 14.2$; $p < 0.05$), while the influence of transport temperature was only found in depurated clams for A.E.C. ($F = 6.2$; $p < 0.05$) and K-value ($F = 4.7$; $p < 0.05$; after one day of transport in *R. philippinarum* and two days of transport in *V. pullastra*), as well as in non-depurated clams ($F = 26.2$, $p < 0.05$; after one day of transport in *V. pullastra* and three days of transport in *R. philippinarum*) (Table 2; Fig. 6).

Concerning correlations, apart from those described previously, *R. phillippinarum*, statistically positive linear relationships were found between glycogen and AMP (clams transported at 22 °C), IMP (all treatments, except T4), Hx (T4), as well as negative linear relationship with ATP (T22). In contrast, for *V. pullastra* a significant positive linear relationship were found between glycogen and ADP (T22), as well as negative linear relationships with IMP (all treatments, except DT4), HxR (clams transported at 22 °C), Hx (all treatments, except DT4) and K-value (clams transported at 22 °C).

3.2.3. Glycogen

In all treatments, glycogen content generally decreased, but was always significantly higher in *V. pullastra* than in *R. philippinarum* (Fig. 9), either depurated ($F = 260.0$, $p < 0.05$) or non-depurated ($F = 107.1$, $p < 0.05$) (Table 2). During depuration, glycogen levels in the adductor muscle of clams decreased in native clams from 2.0 to 1.6 g 100 g^{-1} ww, while in exotic clams the decrease was not statistically different (Fig. 9).

Regarding transport temperatures, significantly lower glycogen levels were observed with both clam species at 22 °C compared to 4 °C, except at one day transport in *V. pullastra*. A significant influence of transport temperature was only found in depurated clams ($F = 45.7$, $p < 0.05$; Table 2), particularly after one day transport in *R. philippinarum*

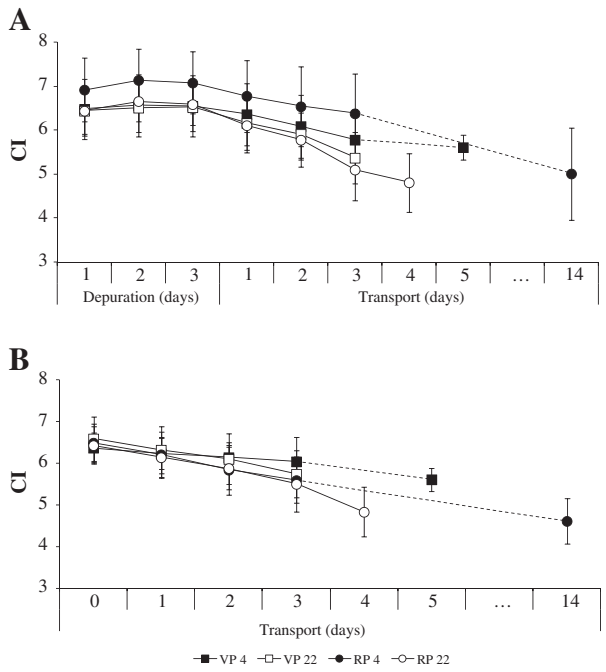


Fig. 4. Condition index (CI) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C. The dashed line indicates that no sampling was performed in these days. Values represent mean ± SD (n = 25). Abbreviations: CI, condition index.

and three days transport in *V. pullastra* (Fig. 9a). The evolution of glycogen contents in both clam species was more pronounced during transport at 22 °C and significantly decreased until reaching LT50 (Fig. 9). At low temperature (4 °C), native clams did not reveal statistical changes in glycogen values throughout transport in both treatments, whereas exotic clams revealed a significant decrease, attaining at LT50 levels of 0.09 and 0.14 g 100 g^{-1} ww in depurated and non-depurated clams, respectively.

4. Discussion

4.1. Survival

The longer survival of *R. philippinarum* exposed to air and maintained at low temperatures compared to *V. pullastra* is likely related to the exotic species' higher adaptability and tolerance to environmental changes, confirming good tolerance of this specie to hypoxic conditions (Ali and Nakamura, 1999, 2000; Ali et al., 1999, Uzaki et al., 2003). Similar findings were highlighted by Widdows et al. (1979) in *Mytilus edulis*, *Mytilus galloprovincialis* and *Cardium edule*.

Differences in survival rate between exotic and native clams can thus be related to distinct physiological conditions that result in different adaptations to habitats in Tagus estuary. *R. philippinarum* usually burrows in sediment below mid-tide level up to 4 m deep and is mostly distributed in the upstream part of the estuary, being subjected to large variations of environmental parameters (Jones et al., 1993). In contrast, *V. pullastra* usually burrows 5 cm in the sediment and is distributed in the mouth of the Tagus estuary, in deeper waters up to 40 m, where animals face lower variations of environmental parameters (FAO, 2012b). According to the findings of Ali et al. (1999), *R. philippinarum* can partially change their aerobic metabolism to anaerobic metabolism. However, clams tightly close the valves during air exposure, revealing that the clams could not survive in such anaerobic conditions. This indicates that air breathing is an important part of the strategy of exotic clams for longer survival in air exposure.

Overall, both clam survival was not negatively affected by depuration. Several studies also reported the absence of mortalities during depuration of several clam species like *Paphia undulata* (El-Gamal, 2011) and *Chamelea gallina* (Maffei et al., 2009). The depuration process even enabled additional two days storage extension of both clam species until reaching LT50, which has an economical importance to stakeholders along the trade chain. The results also demonstrated that lower transport temperature (4 °C) was able to prolong around

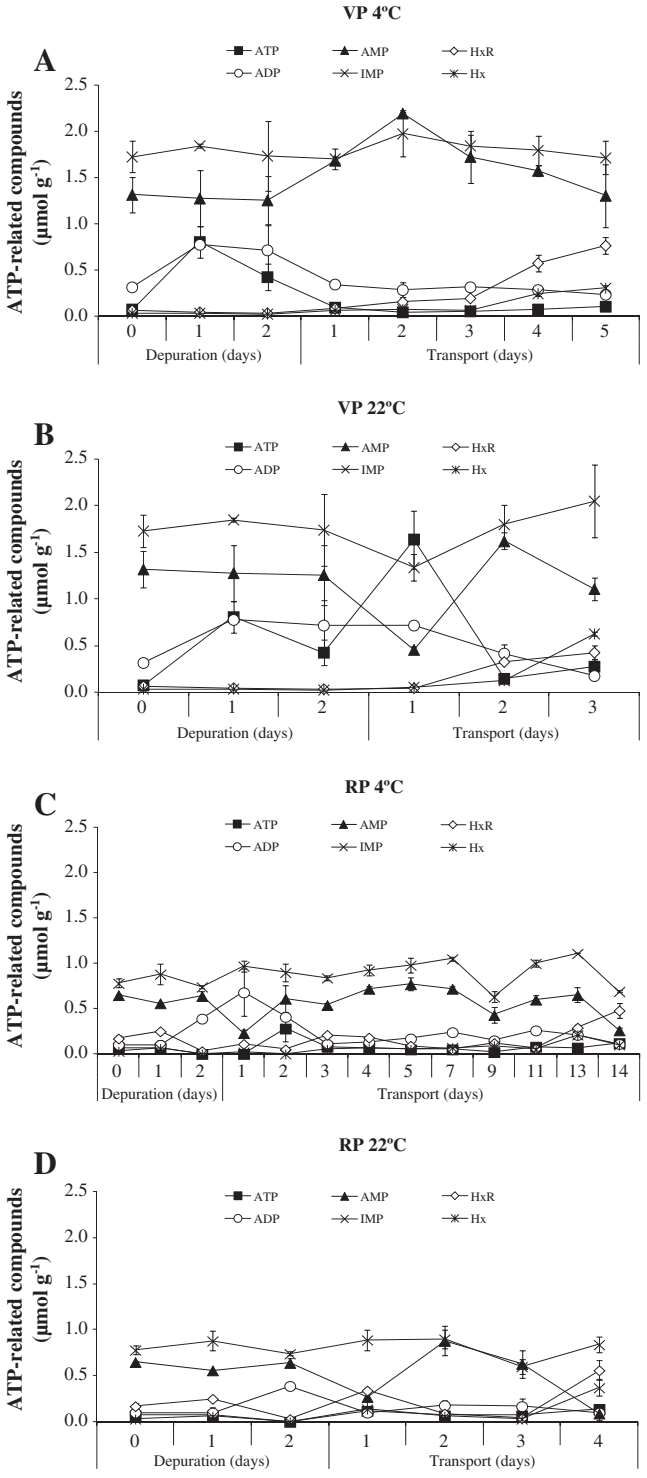


Fig. 5. Changes in levels of ATP-related compounds (µmol g^{-1}, wet weight) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to depuration followed by transported after at 4 or 22 °C. Values represent mean ± SD (n = 3). Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate, AMP, adenosine 5′-monophosphate, IMP, inosine 5′-monophosphate, HxR, inosine; Hx, hypoxanthine.

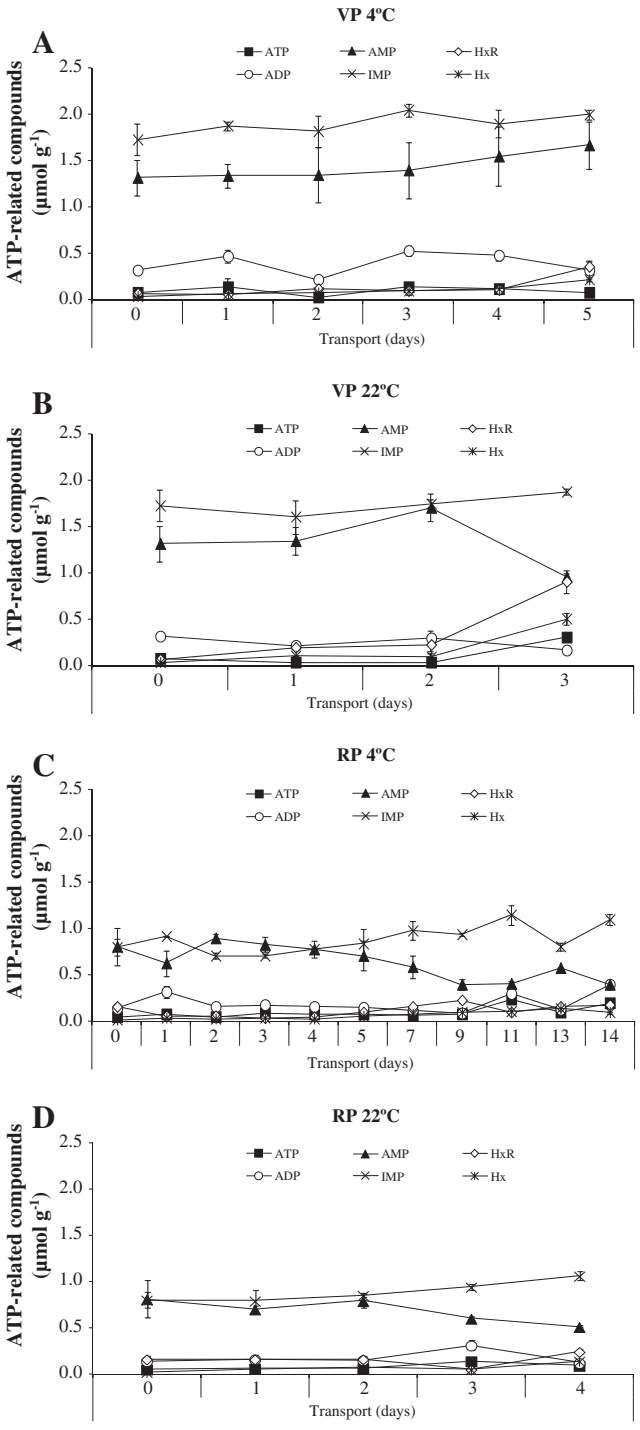


Fig. 6. Changes in levels of ATP-related compounds (µmol g^{-1}, wet weight) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to semi-dry transport at 4 or 22 °C without depuration. Values represent mean ± SD (n = 3). Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate, AMP, adenosine 5′-monophosphate, IMP, inosine 5′-monophosphate, HxR, inosine; Hx, hypoxanthine.

2 and 10 days *V. pullastra* and *R. philippinarum* survival, respectively. It is known that bivalves can survive out of water for extended periods if kept in a cold and moist environment, since such conditions enable a reduction of bivalve's metabolism and consequently the decrease of excretion products. Additionally, the growth of microorganisms is also inhibited as well as enzymatic deterioration reactions and food spoilage (Sea Grant, 2012).

Table 5
Results of two-way ANOVA evaluating the effects of transport temperature (4 and 22 °C) and species (*Venerupis pullastra* and *Ruditapes philippinarum*) on ATP-related compounds (IMP, ATP, ADP, AMP, Hx and HxR).

	Depurated				Non-depurated			
	df	MS	F	p	df	MS	F	p
ATP								
Temperature (T)	1	1.2	10.3	0.002*	1	0.020	2.4	0.129
Species (S)	1	1.1	9.6	0.003*	1	0.000	0.001	0.980
T × S	1	1.2	10.8	0.002*	1	0.001	0.154	0.696
Error	52	0.115			52	0.008		
ADP								
Temperature (T)	1	0.000	0.012	0.914	1	0.080	8.8	0.004*
Species (S)	1	0.267	7.9	0.007*	1	0.045	4.9	0.030*
T × S	1	0.493	14.6	0.000*	1	0.059	6.5	0.014*
Error	52	0.034			52	0.009		
AMP								
Temperature (T)	1	0.223	5.7	0.021*	1	0.030	5.3	0.025*
Species (S)	1	2.3	59.0	0.000*	1	0.814	144.0	0.000*
T × S	1	0.346	8.9	0.004*	1	0.011	2.0	0.167
Error	52	0.039			52	0.006		
IMP								
Temperature (T)	1	0.005	0.109	0.743	1	0.004	0.341	0.562
Species (S)	1	13.2	285.4	0.000*	1	13.2	1 154.3	0.000*
T × S	1	0.254	5.5	0.023*	1	0.305	26.6	0.000*
Error	52	0.046			52	0.012		
HxR								
Temperature (T)	1	0.073	6.0	0.018*	1	1.3	17.8	0.000*
Species (S)	1	0.057	4.7	0.036*	1	1.9	26.3	0.000*
T × S	1	0.009	0.735	0.396	1	0.136	1.8	0.181
Error	52	0.012			52	0.074		
Hx								
Temperature (T)	1	1.7	7.6	0.008*	1	0.248	5.3	0.025*
Species (S)	1	5.0	22.1	0.000*	1	3.0	64.1	0.000*
T × S	1	0.330	1.4	0.233	1	0.018	0.379	0.540
Error	52	0.226			52	0.047		

Abbreviations: Asterisk indicates significant differences.

4.2. Physiological analyses

4.2.1. Condition index

CI has been used as an ecophysiological measure of bivalve health status, summarizing the physiological/nutritional status under changed environmental conditions (Mubiana et al., 2006) and also provides a comprehensive picture of meat quality for bivalve market value (Orban et al., 2004). The initial variations of CI found between both clam species reflect different environmental (usually more extreme for exotic clams) and physiological demands. It was also found that transport temperature strongly influences CI, but not depuration. Stressful conditions might be responsible by CI reduction observed during transport, namely the lack of feed. Previous studies reported that bivalve condition is usually associated with the utilization of biochemical reserves that decrease body weight (Albentosa et al., 2007; Hummel et al., 1989). So, the lowest CI at LT50 was detected when glycogen reserve also reached the lowest values. On the other hand, prolonged periods of very low oxygen that occurs during transport, particularly at high temperature, can stress bivalves, causing them to gape out of water inside the mantle cavity, which leads to a decrease in CI and consequently the animal death (Laing and Spencer, 2006). Our study was in accordance with the findings of Pampanin et al. (2005), which considered CI and survival as good physiological indicators of the health status of air exposed mussel *M. galloprovincialis*.

4.2.2. Nucleotides

Nucleotides and related compounds generally play an important role as coenzymes. They participate actively in muscle metabolism and supply energy to physiological processes (Caballero, 2009). The

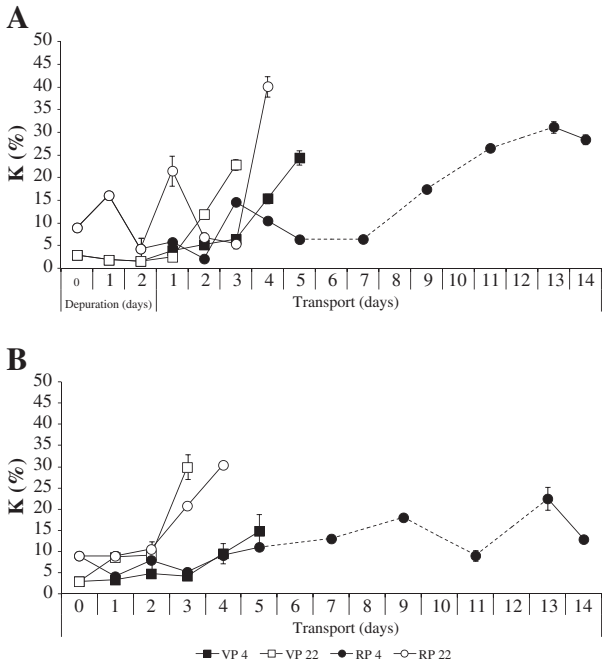


Fig. 7. K-value (%) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C. Values represent mean ± SD (n = 3). The dashed line indicates that no sampling was performed in these days.

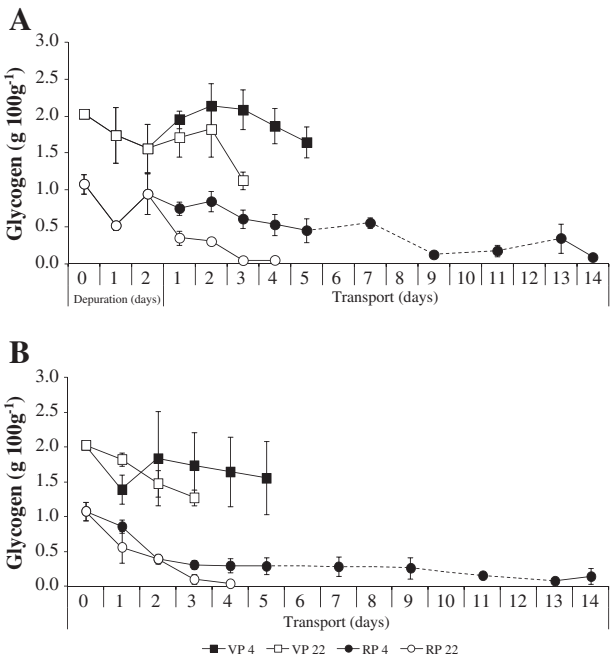


Fig. 9. Glycogen content (g 100 g^{-1}, wet weight) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C. Values represent mean ± SD (n = 3). The dashed line indicates that no sampling was performed in these days.

results obtained in this study suggested the following pathway of ATP degradation: ATP → ADP → AMP → IMP → HxR → Hx. The compounds IMP and AMP were detected at high concentrations during depuration and transport, whereas ATP levels were very low, thereby remaining throughout the transport period. The initial catabolism of ATP by endogenous enzymes in several fish and molluscs normally results in a temporary accumulation of IMP (Olafsdóttir et al., 1997). Mendes et al. (2001) reported that AMP is a dominant nucleotide of molluscs and crustaceans, and its accumulation is the result of a highly reduced or nonexistent AMP deaminase activity. The degradation of ATP to IMP is mainly attributed to the endogenous autolytic enzyme activity (Gram and Huss, 1996), whereas the breakdown from IMP to HxR and Hx is connected with the enzymatic activity and bacterial growth (Surette et al., 1988). Initially, HxR and Hx contents were very low, but increased during the transport period, particularly at 22 °C. Thus, the accumulation of HxR and Hx can be used as a stress indicator, due to their formation during the simulated transport period. The ratio of the sum of these compounds to the total sum of the ATP breakdown products, expressed as a percentage, is known as the K-value (Saito et al., 1959). Since this index followed the same trend of HxR and Hx, K-value can also be considered as a stress indicator for both clam species.

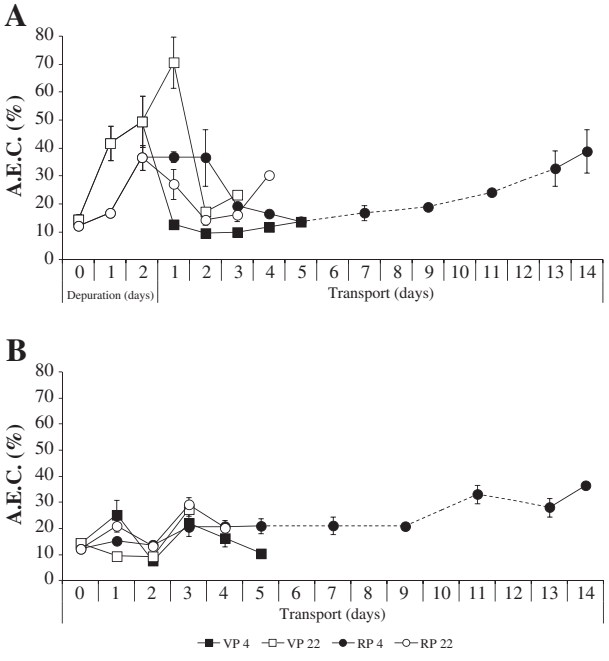


Fig. 8. A.E.C. value (%) of native (*Venerupis pullastra*, VP) and exotic clams (*Ruditapes philippinarum*, RP) subjected to different treatments: A) depuration and semi-dry transport at 4 or 22 °C; B) non-depurated clams subjected to semi-dry transport at 4 or 22 °C. Values represent mean ± SD (n = 3). The dashed line indicates that no sampling was performed in these days.

HxR and Hx showed a positive and significant correlation with survival rate in almost all treatments of both clam species, which supports the fact that ATP degradation occurred as mortalities increased. Overall, clams transported at 22 °C showed higher contents of Hx than HxR, and lower AMP levels, indicating that ATP catabolism and its breakdown products were accelerated and affected by the physiological status of clams transported at higher temperatures, which is in accordance with the findings of several studies (Hattula, 1997; Ponce de Leon et al., 1994). The higher levels of AMP, IMP, HxR, Hx and the sum of ATP breakdown products found in *V. pullastra* at LT50 reflects the rapid consumption of ATP energy in native clams. This result suggests that the rate of nucleotide catabolism varies according to species and specimen physiological conditions.

It is known that A.E.C. value strongly varies in invertebrates according to the importance of the internal or external stressors (natural or anthropogenic) (Picado and Le Gal, 1999). The more stressed an animal becomes, the more energy it uses, thus lowering its AEC level (Maguire et al., 1999). In this study, A.E.C. results indicated that during depuration both clam species were not able to recover from the initial harvesting sub-optimal stressful conditions (levels below 50%), and

consequently during transport maintained stressful conditions (Bayne et al., 1985). A.E.C. value was not a very accurate physiological marker for *R. philippinarum*, since the values generally increased during transport. Similarly, other researchers have concluded that A.E.C. is not a good index of the physiological condition of several bivalve species (Da Ros et al., 2003; Marin et al., 2005).

4.2.3. Glycogen

The relative importance of energy reserves and their order of utilization vary among bivalve species (Du and Mai, 2004). Strategies of fuel reserve usage may change depending on species, starvation/food availability, sexual maturation and developmental stage, being selected to prolong the survival of the organisms (Dridi et al., 2007; Kang et al., 2000).

The higher glycogen levels observed in *V. pullastra* compared to *R. philippinarum* can thus be related to different physiological requirements. Indeed, LT50 was attained in native clams when glycogen content was about 1.1–1.7 g 100 g^{-1} ww, while glycogen levels in exotic clams were almost completely depleted at LT50. Such results corroborate the findings of Albentosa et al. (2007) that highlighted that *V. pullastra* obtains energy mainly from catabolism of proteins and lipids, whereas another *Ruditapes* species (*Ruditapes decussatus*) obtains a different energy source equally from proteins and carbohydrates. In the juvenile clam *R. philippinarum*, 75% of total carbohydrates are used over a 35-day starvation period (Laing, 1993). In *Crassostrea gigas*, Riley (1976) observed that the level of glycogen dropped throughout the longest starvation period as opposed to a slight decrease for lipid and protein.

Our results also clearly demonstrate that *V. pullastra* maintained at 4 °C seems not to use their metabolic reserves, in contrast with clams transported at 22 °C as well as *R. philippinarum*. Several authors (Albentosa et al., 2007; Hummel et al., 1989) have described that in some bivalves like mussels, cockles and clams, glycogen is the first biochemical reserve consumed few days after starvation. Under stressful conditions like semi-dry transport, glycogen levels are used for maintenance needs and reflect bivalve capacity to sustain further stress (Patrick et al., 2006). Indeed, Hummel et al. (1989) reported a latent period at 5 °C, in which mussels (*M. edulis*) and cockles (*Cerastoderma edule*) do not use their reserves, followed by a strong decrease in glycogen levels after 7 days of air exposure. Reduction or depletion in metabolic reserves like glycogen is often associated with mass mortalities of adult *Crassostrea gigas* (Li et al., 2007; Patrick et al., 2006), *Macoma balthica* (Hummel et al., 2000) and *R. philippinarum* (Robert et al., 1993; Uzaki et al., 2003).

According to the current findings glycogen seems to be a good indicator of the physiological condition of clams, as good correlation was found between with clam survival and CI. Similar conclusion was reached by Uzaki et al. (2003) for *R. philippinarum* when this clam specie is exposed to oxygen-deficient waters.

5. Conclusions

This is the first study where a comparison was made between two clam species, *V. pullastra* (native) and *R. philippinarum* (exotic) from Tagus estuary, to assess their physiological responses during depuration and transport. The results demonstrate the higher survival of the exotic clam species compared to native populations when exposed to air. Additionally, depuration does not negatively affect native or exotic clams from Tagus estuary, and it even improves their physiological condition and enables the shelf-life extension for 2 days. The best semi-dry transport/storage conditions to maintain high quality clams should be performed at low temperatures (4 °C). CI, nucleotides and glycogen content are good and useful indicators of the physiological status of clams along the trade chain. *V. pullastra* and *R. philippinarum* showed a similar pattern in these parameters, but in different levels: native clams generally revealed higher condition index and glycogen content, as well as lower nucleotide ratio (K-value) and A.E.C. value than exotic clams, which indicate that native and exotic clams have different physiological requirements.

All the physiological responses measured in the present study give a detailed and accurate outline of the conditions of clams during trade chain. Overall, this study is particularly useful for aquaculture management and strategy since the physiological tests measure the quality of marketed products, characterize the apparent "health" of cultured stocks and reflect the ability of bivalves to withstand adverse natural and/or anthropogenic stress.

Acknowledgments

This study was supported by the SHELLPLANT research project (ref. 232273) funded by the European Commission (FP7). The first author acknowledges a PhD scholarship of the Portuguese Foundation for Science and Technology (FCT) (Ref. SFRH/BD/69274/2010), whereas the last two authors were supported by a research contract from the Ciência 2007 and 2008 Programmes of FCT.

References

Albentosa, M., Fernández-Reiriz, M.J., Labarta, U., Pérez-Camacho, A., 2007. Response of two species of clams, *Ruditapes decussatus* and *Venerupis pullastra*, to starvation: physiological and biochemical parameters. Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology 146, 241–249.
Ali, F., Nakamura, K., 1999. Effect of temperature and relative humidity on the tolerance of the Japanese clam, *Ruditapes philippinarum* (Adams & Reeve), to air exposure. Aquaculture Research 30, 629–636.
Ali, F., Nakamura, K., 2000. Metabolic characteristics of the Japanese clam *Ruditapes philippinarum* (Adams & Reeve) during aerial exposure. Aquaculture Research 31, 157–165.
Ali, F., Nakamura, K., Yamasaki, S., 1999. Physiological characteristics of aerial survival in Japanese clam, *Ruditapes philippinarum* (Adams and Reeve). Suisan zoshoku 47 (3), 381–389.
Atkinson, D.E., 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry 7, 4030–4034.
Bayne, B.L., Brown, D.A., Burns, K., Dixon, D.R., Ivanovici, A., Livingstone, D.R., Lowe, D.M., Moore, M.N., Stebbing, A.R.D., Widdows, J., 1985. The Effects of Stress and Pollution on Marine Animals. Praeger Scientific, New York (384 pp.).
Brogueira, M.J., Cabeçadas, G., 2006. Identification of similar environmental areas in Tagus estuary by using multivariate analysis. Ecological Indicators 6, 508–515.
Caballero, B., 2009. Guide to Nutritional Supplements. Elsevier Ltd., Oxford, UK (548 pp.).
Da Ros, L., Nesto, N., Nasci, C., Moschino, V., Pampanin, D., Marin, M.G., 2003. Biochemical and behavioural effects of hydraulic dredging on the target species *Chamelea gallina*. Fisheries Research 64, 71–78.
Dridi, S., Romdhane, M.S., Elcafsi, M.H., 2007. Seasonal variation in weight and biochemical composition of the Pacific oyster, *Crassostrea gigas*, in relation to the gametogenic cycle and environmental conditions of the Bizert lagoon, Tunisia. Aquaculture 263, 238–248.
Du, S.B., Mai, K.S., 2004. Effects of starvation on energy reserves in young juveniles of abalone *Haliotis discus hannai* Ino. Journal of Shellfish Research 23, 1037–1039.
El-Gamal, M.M., 2011. The effect of depuration on heavy metals, petroleum hydrocarbons, and microbial contamination levels in *Paphia undulata* (Bivalvia: Veneridae). Czech Journal of Animal Science 56 (8), 345–354.
El-Shenawy, N.S., 2004. Heavy metal and microbial depuration of the clam *Ruditapes decussatus* and its effect on bivalve behavior and physiology. Environmental Toxicology 19, 143–153.
FAO, 2012a. Fisheries and Aquaculture Information and Statistics Service. (Retrieved September, 21, 2012 from) http://www.fao.org/fishery/statistics/en.
FAO, 2012b. Species Fact Sheets: *Venerupis pullastra* (Montague, 1803). (Retrieved September, 21, 2012 from) http://www.fao.org/fishery/culturedspecies/Venerupis_pullastra/en.
Flassch, J.P., Leborgne, Y., 1992. Introduction in Europe, from 1972 to 1980, of the Japanese Manila clam (*Tapes philippinarum*) and the effects on aquaculture production and natural settlement. ICES Marine Science Symposia 194, 92–96.
Freitas, R., Pinto, L.R., Sampaio, M., Costa, A., Silva, M., Rodrigues, A.M., Quintino, V., Figueira, E., 2012. Effects of depuration on the element concentration in bivalves: comparison between sympatric *Ruditapes decussatus* and *Ruditapes philippinarum*. Estuarine, Coastal and Shelf Science 110, 43–53.
Gaspar, M.B., 2010. Distribuição, abundância e estrutura demográfica da amêijoa-japonesa (*Ruditapes philippinarum*) no Rio Tejo. Relatório do IPIMAR 1–6.
Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology 33, 121–137.
Hattula, T., 1997. Adenosine Triphosphate Breakdown Products as a Freshness Indicator of Some Fish Species and Fish Products, VTT Publications 297. VTT (Technical Research Centre of Finland), Espoo (48 pp.).
Hummel, H., Wolf, L., Zurburg, W., Apon, L., Bogaards, R.H., 1989. The glycogen content in stressed marine bivalves: the initial absence of a decrease. Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology 94 (4), 729–733.

Hummel, H., Bogaards, R.H., Bachelet, G., Caron, F., Sola, J.C., Amiard-Triquet, C., 2000. The respiratory performance and survival of the bivalve *Macoma balthica* (L.) at the southern limit of its distribution area: a translocation experiment. Journal of Experimental Marine Biology and Ecology 251, 85–102.

Jones, G.G., Sanford, C.L., Jones, B.L., 1993. Manila Clams: Hatchery and Nursery Methods. Innovative Aquaculture Products Ltd. and Science Council of British Columbia, Victoria, BC (73 pp.).

Kang, C.K., Park, M.S., Lee, P.Y., Choi, W.J., Lee, W.C., 2000. Seasonal variations in condition, reproductive activity, and biochemical composition of the Pacific oyster, *Crassostrea gigas* (Thunberg), in suspended culture in two coastal bays of Korea. Journal of Shellfish Research 19, 771–778.

Laing, I., 1993. The response of Manila clam, *Tapes phillippinarum*, juveniles to nutritive stress. Journal of Experimental Marine Biology and Ecology 173, 111–121.

Laing, I., Spencer, B.E., 2006. Bivalve cultivation: criteria for selecting a site. Cefas Science Series Technical Report No. 136. (34 pp.).

Lee, S.Y., 1996. Distribution pattern and interaction of two infaunal bivalves, *Tapes philippinarum* (Adams & Reeve) and *Anomalocardia squamosa* (Linnaeus) (Bivalvia: Veneridae). Journal of Experimental Marine Biology and Ecology 201, 253–273.

Lee, R., Lovatelli, A., Ababouch, L., 2008. Bivalve depuration: fundamental and practical aspects. FAO Fisheries Technical Paper. No. 511.FAO, Rome (161 pp.).

Li, Y., Qin, J.G., Abbott, C.A., Li, X., Benkendorff, K., 2007. Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas*: an explanation for summer mortality in Pacific oysters. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 293, 2353–2362.

Maffei, M., Vernocchi, P., Lanciotti, R., Guerzoni, M.E., Belletti, N., Gardini, F., 2009. Depuration of striped Venus clam (*Chamelea gallina* L.): effects on microorganisms, sand content, and mortality. Journal of Food Science 74 (1), M1–M7.

Maguire, J.A., Fleury, P.G., Burnell, G.M., 1999. Some methods for quantifying quality in the scallop *Pecten maximus* (L.). Journal of Shellfish Research 18, 59–66.

Marin, M.G., Moschino, V., Meneghetti, F., Da Ros, L., 2005. Effects of mechanical stress in under-sized clam, *Tapes philippinarum*: a laboratory approach. Aquaculture International 13, 75–88.

Mendes, R., Quinta, R., Nunes, M.L., 2001. Changes in baseline levels of nucleotides during ice storage of fish and crustaceans from the Portuguese coast. European Food Research and Technology 212, 141–146.

Mubiana, V.K., Vercauteren, K., Blust, R., 2006. The influence of body size, condition index and tidal exposure on the variability in metal bioaccumulation in *Mytilus edulis*. Environmental Pollution 144, 272–279.

Nie, Z.Q., 1991. The culture of marine bivalve mollusks in China. In: Menzel, W. (Ed.), Estuarine and Marine Bivalve Mollusks Culture. CRC Press, Boston, pp. 261–276.

Olafsdóttir, G., Martinsdóttir, E., Oehlenschläger, J., Dalgaard, P., Jensen, B., Undeland, I., Mackie, I.M., Henehan, G., Nielsen, J., Nielsen, H., 1997. Methods to evaluate fish freshness in research and industry. Trends in Food Science and Technology 8, 258–265.

Orban, E., Di Lena, G., Masci, M., Nevigato, T., Casini, I., Caproni, R., Gabelli, L., Pellizzato, M., 2004. Growth, nutritional quality and safety of oysters (*Crassostrea gigas*) cultured in the lagoon of Venice (Italy). Journal of the Science of Food and Agriculture 84, 1929–1938.

Pampanin, D.M., Volpato, E., Marangon, I., Nasci, C., 2005. Physiological measurements from native and transplanted mussel (*Mytilus galloprovincialis*) in the canals of Venice. Survival in air and condition index. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 140, 41–52.

Patrick, S., Faury, N., Goulletquer, P., 2006. Seasonal changes in carbohydrate metabolism and its relationship with summer mortality of Pacific oyster *Crassostrea gigas* (Thunberg) in Marennes-Oleron bay (France). Aquaculture 252, 328–338.

Picado, A., Le Gal, Y., 1999. Adenylate energy charge as a tool for evaluating sub-lethal toxicity. Qatar University Science Journal 19, 36–45.

Ponce de Leon, S., Inoue, N., Shinano, H., 1994. Shelf life of sardine and bacterial flora in brine containing acetic acid during immersed storage. Fisheries Science 60, 429–433.

Riley, R.T., 1976. Changes in the total protein, lipid, carbohydrate, and extracellular body fluid free amino acids of the Pacific oyster *Crassostrea gigas*, during starvation. Proceedings of the National Shellfish Association 65, 84–90.

Robert, R., Trut, G., Laborde, J.L., 1993. Growth, reproduction and gross biochemical composition of the Manila clam, *Ruditapes philippinarum*, in the Bay of Arcachon, France. Marine Biology 116, 291–299.

Ryder, J.M., 1985. Determination of adenosine triphosphate and its breakdown products in fish muscle by high-performance liquid chromatography. Journal of Agricultural and Food Chemistry 33, 678–680.

Saito, T., Arai, K., Matsuyoshi, M., 1959. A new method for estimating the freshness of fish. Bulletin of the Japanese Society of Scientific Fisheries 24, 749–750.

Sea Grant, 2012. Managing the Cold Chain for Quality and Safety. (Retrieved September, 21, 2012 from) http://seafood.ucdavis.edu/pubs/coldchain.doc.

Solidoro, C., Pastres, R., Melaku Canu, D., Pellizzato, M., Rossi, R., 2000. Modelling the growth of *Tapes philippinarum* in Northern Adriatic lagoons. Marine Ecology Progress Series 199, 137–148.

Surette, M.E., Gill, T.A., LeBlanc, P.J., 1988. Biochemical basis of postmortem nucleotide catabolism in cod (*Gadus morhua*) and its relationship to spoilage. Journal of Agricultural and Food Chemistry 36, 19–22.

Uzaki, N., Kai, M., Aoyama, H., Suzuki, T., 2003. Changes in mortality rate and glycogen content of the Manila clam *Ruditapes philippinarum* during the development of oxygen-deficient waters. Fisheries Science 69, 936–943.

Viles, P., Silverman, J., 1949. Determination of starch and cellulose with anthrone. Journal of Analytical Chemistry 21, 950–953.

Widdows, J., Bayne, B.L., Lyvingstone, D.R., Newell, R.I.E., Donkin, P., 1979. Physiological and biochemical responses of bivalve molluscs to exposure to air. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 62 (2), 301–308.

CHAPTER 6

Effects of depuration on metal levels and health status of bivalve molluscs

Anacleto P., Maulvault A. L., Nunes M. L., Carvalho M. L., Rosa R. and Marques A.

In press in *Food Control*

Effects of depuration on metal levels and health status of bivalve molluscs

Patrícia Anacleto[a,b,c,*], Ana Luísa Maulvault[a,c], Maria Leonor Nunes[a,c], Maria Luísa Carvalho[d], Rui Rosa[b], António Marques[a,c]

[a] Division of Aquaculture and Upgrading (DivAV), Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.), Avenida de Brasília, 1449-006 Lisbon, Portugal
[b] Guia Marine Laboratory, Center of Oceanography, Faculty of Sciences (FCUL), University of Lisbon, Campo Grande, 1749-016 Lisbon, Portugal
[c] Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal
[d] Center of Atomic Physics, Physics Department, Faculty of Sciences and Tecnology, New University of Lisbon, 2829-516 Caparica, Portugal

ARTICLE INFO

Keywords:
Depuration
Bivalves
Mortality
Glycogen
Toxic elements
Macro and trace elements

* Corresponding author.
Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.),
Avenida de Brasília, 1449–006, Lisboa, Portugal.
Tel.: +351 213 027 025; Fax: +351 21 3015948
E-mail address: panacleto@ipma.pt (P. Anacleto)

ABSTRACT

Depuration has been mandatory to eliminate microorganisms in bivalve molluscs, but not to eliminate toxic chemicals, since there is still shortage of scientific evidence about the efficacy of depuration to decrease the levels of toxic chemicals in bivalves. This study aims to assess the effects of depuration on the levels of toxic, macro and trace elements in three bivalve species (*Ruditapes philippinarum*-RP, *Mytilus galloprovincialis*-MG and *Scrobicularia plana*-SP), taking into account their condition. During depuration, a significant reduction of elements occurred in RP (Fe, Pb, Cu, Rb, Br, Hg, Cd and As), MG (Cl, Sr, Fe, Pb and Br) and SP (Fe, Pb, Cu and Rb), while an increase was registered for other elements in RP (Cl and Sr), MG (S and Zn) and SP (Cl, Zn, Br and Sr). After two days of depuration, Pb in SP had decreased to levels below the permissible limits, thus allowing this species to be acceptable for human consumption as far as toxic elements are concerned. SP revealed the highest glycogen levels compared to the other species indicating different physiological requirements. However, in this species glycogen levels significantly decreased after two days of depuration and mortality increased. In contrast, in MG and RP glycogen and mortality did not vary significantly during depuration. Therefore, SP should be only depurated for a maximum period of two days. Overall, the results of this study are particularly useful to retailers to ensure high quality bivalve products for consumers. Additionally, glycogen can be used as a suitable biomarker of healthy status of bivalve species during depuration.

1. Introduction

Chemical pollution in shellfish-growing waters is a worldwide problem in coastal areas (Almeida, & Soares, 2012). Bivalve molluscs (e.g. mussels, oysters and clams) can take up contaminants from sediments, suspended particulate materials, water column and also food sources (Laffon, Rábade, Pásaro, & Méndez, 2006; Livingstone, 1993). They have been widely used for many years as bioindicator (sentinel) organisms in monitoring of chemical pollutants and biomonitoring (estimation of environmental quality) in aquatic ecosystems. This is particularly due to their sedentary nature or immobility, filter-feeding activity, low metabolism, contact with sediments, suitable size for biochemical analysis, wide distribution in marine, estuarine and freshwater environments, practicality in collection, ability to bioaccumulate pollutants and high tolerance to chemical exposure due to a remarkably active immune system (Emmanouil, Kypriotakis, Kungolos, & Machera, 2008; Gupta, & Singh, 2011; Waykar, & Deshmukh, 2012; Zuykov, Pelletier, & Harper, 2013). Among environmental contaminants, toxic elements are a main concern due to their harmful effects on organisms and ability to bioaccumulate in aquatic ecosystems (Censi *et al.*, 2006). Several bivalve species live in estuaries that are subjected to several anthropogenic pressures, thus being exposed to high levels of toxic elements (Förstner, & Wittmann, 1979). The incorporation rate of contaminants in bivalves depends on biotic factors (e.g. species, age, sex, soft-body weight, gametogenesis and physiological status) and abiotic factors (e.g. availability of contaminants in the environment, filtration rate, temperature, salinity, pH, chemical species and interaction with other elements) (Fernández-Tajes *et al.*, 2011; Philips, 1980).

Elements are separated into two categories: essential and non-essential. Essential elements, such as Cu, Fe, Mn, Sr, Se and Zn, have defined biological functions in organisms (Simkiss, 1981; Williams, 1981), whereas non-essential elements, such as Hg, Pb, Cd and As seem not to participate in any metabolic functions (Dallinger, 1995; Suzuki, & Suzuki, 1996). The latter elements are among the main toxic elements found in water bodies and may accumulate in bivalve species at high concentrations that can reach several orders of magnitude above those in the environment (Fang, Cheung, & Wong, 2003; Zuykov *et al.*, 2013) and may be biomagnified in the food chain to levels that cause physiological impairment at higher trophic levels and in consumers (Lemiere *et al.*, 2005; Raposo *et al.*, 2009). The routes of transmission from the environment to humans include the consumption of raw or lightly/extensively cooked shellfish, representing a significant human health hazard to consumers (Lees, Younger, & Dore, 2010). Nonetheless, the European Commission has set Maximum Permissible Limits (MPLs) for toxic elements in edible tissues of bivalve molluscs: 0.5 mg/kg for Hg, 1.0 mg/kg for Cd and 1.5 mg/kg for Pb (EC, 2006). Currently, *S. plana* from Tagus estuary is an example of bivalve species declared unfit for human consumption due to the high levels of Pb often found above the MPLs (DR, 2013). Between 2000 and 2010, 6.7% of all notifications from the European Union Rapid Alert System for Food and Feed (RASFF) were recorded for bivalve molluscs contaminated with toxic elements (RASFF, 2012; SARF, 2010).

Depuration is currently mandatory in the EU to diminish pathogenic microorganisms' levels (e.g. *Escherichia coli*) in bivalves harvested for human consumption in polluted waters (B category) in order to ensure healthy and safe products for commercialization (Regulation EC Nos. 853/2004 and 854/2004; EC, 2004a, 2004b). This method consists in maintaining bivalves up to 48 h in sterile seawater (through ozone, UV-light, chlorination or iodophors) with sufficient oxygen and without any feed (Lee, Lovatelli, & Ababouch, 2008). Several factors may influence the efficacy of depuration, such as: the system design, initial water quality, oxygenation and flow rates, salinity, temperature, shellfish-to-water ratios, removal and settlement of faecal material, type and amount of pollutants and the

duration (Barile *et al.*, 2009; Cozzi, Suffredini, Ciccaglioni, & Croci, 2009; Lee, & Younger, 2002; Manfra, & Accorneo, 2005). Although, depuration is effective to remove faecal bacterial contaminants from bivalves, the effectiveness of depuration to eliminate chemical contaminants is still poorly understood, despite few studies reported lower levels of some toxic metals in bivalves after depuration (e.g. El-Gamal, 2011).

The physiological and biochemical responses of bivalve species to depuration are also poorly understood. Commonly, condition index (CI) and glycogen content are good physiological/biochemical parameters to assess the health status of bivalves. CI has long been used for biological and commercial purposes (Baird, 1958; Venkataraman, & Chari, 1951) and is also recognized as a useful biomarker to reflect the ability of bivalves to withstand adverse natural and/or anthropogenic stressors (Bressan, & Marin, 1985; Fernandez-Castro, & De Vido de Mattio, 1987; Mann, 1978). Glycogen represents the most prominent carbohydrate stored in marine bivalves and is commonly used as an indicator of their nutritional status (Barber, & Blake, 1981).

In this context, the aims of the present study were to evaluate the effects of depuration on the potential accumulation/removal of toxic elements (Hg, Cd, Pb and As) and macro/trace elements (S, Cl, K, Ca, Fe, Zn, Br, Cu, Se, Rb and Sr) in three bivalve species (*Ruditapes philippinarum*, RP, *Mytilus galloprovincialis*, MG and *Scrobicularia plana*, plana, SP) of economical importance in Southern Europe. These species were selected due to their socio-economic relevance (European production amounted to 423 thousand tonnes in 2012; EUROSTAT, 2014), high demand by consumers (Anacleto, Barrento, Nunes, Rosa, & Marques, 2014), as well as to their different feeding strategies, filtration capacities, and habitats (Akberali, & Trueman, 1985). In fact, clams (SP and RP) are burrowing species (Akberali, & Davenport, 1981; Jones, Sanford, & Jones, 1993), while mussels (MG) live attached to rocky shores in intertidal areas (FAO, 2014). Additionally, the animal condition of these bivalve species (survival, condition index and glycogen content) was also investigated during depuration.

2. Materials and methods

2.1. Study area and collection of bivalves

Three bivalve species were collected in April 2012 at different sampling sites of Tagus estuary (38°44′ N, 9°08′ W; Fig. 1). It is one of the largest estuaries on the west coast of Europe in the most populated area of Portugal, with a broad shallow bay covering an area of 320 km² (Brogueira, & Cabeçadas, 2006). The bivalve species were: Japanese carpet shell clam, RP (Adams & Reeve, 1850) harvested near Alcochete (n = 210); Mediterranean mussel, MG (Lamarck, 1819) collected near Algés (n = 210); and peppery furrow shell, SP (Da Costa, 1778) harvested near Sítio das Hortas (n = 210; Fig. 1). The bivalves were cleaned and washed from mud

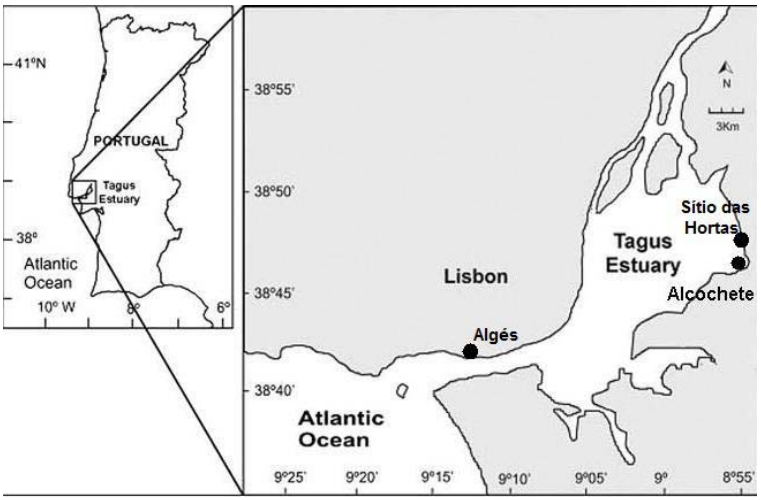


Fig. 1. Map of Portugal with enlarged portion of the Tagus estuary. Dark dots indicate the samplings sites (Alcochete for RP, Algés for MG and Sítio das Hortas for SP).

and sand with seawater, immediately stored at stable conditions (16 °C) and transported in isothermal insulated boxes to the laboratory (approximately two hours of transport period). Biometric data, i.e. total length (mm), width (mm), height (mm), total weight (g) and edible weight (g) were registered from thirty specimens of each species.

2.1.1. Depuration experiment

Depuration was initiated 2 h after bivalves harvesting simulating the commercial practices commonly used (EC, 2004a), and was conducted in three recirculated tanks (width, 31 cm; length, 31 cm; height, 40 cm) for each species, each containing 15 L of UV sterilized and filtered seawater (0.2 µm) taken from open sea in an area not subjected to any anthropogenic pressure and subjected to strong hydro dynamism, likely having lower levels of chemical contaminants than in Tagus estuary. The flow rate entering the circuit was 2.9 L/min. Each tank contained around 2 kg of bivalves, which corresponds to bivalve densities usual in Portuguese depuration facilities. In order to simulate the usual conditions in these facilities, animals were not fed during the experimental period and seawater was kept with continuous aeration and constant temperature (17.6 °C), salinity (35 g/L) and pH (6.98). Thirty specimens from each species (ten from each tank or replicate) were randomly collected at 0, 2, 4, 6 and 8 days of depuration. Behavioural activity was checked using the criteria described by El-Shenawy (2004) with some modifications. Dead specimens were removed and mortality was recorded. Biometric data were also registered at each sampling day for assessment of condition index. The adductor muscle and mantle (including viscera) were separated. Then pooled samples of adductor muscles and mantles, respectively, were made from the ten specimens from each tank. Samples were freeze-dried for 48 h at -50 °C and low pressure (approximately 10^{-1} atm), homogenized with a grinder, vacuum-packed and stored at -80 °C until further analyses. Biochemical analyses were carried out in triplicate

2.2. Condition Index

The Condition Index (CI) was calculated according to the following equation in wet tissue (Maguire, Fleury, & Burnell, 1999):

$$CI = \frac{[total\ weight\ (g)]}{[shell\ lenght] \times [shell\ height] \times [shell\ width](cm)} \times 10{,}000$$

2.3. Glycogen

Glycogen concentrations were determined with a colorimetric method using a solution of anthrone-sulfuric acid (Leyva, Quintana, Sánchez, Rodríguez, Cremata, & Sánchez, 2008; Viles, & Silverman, 1949). Briefly, adductor muscle samples (25 mg dry weight) were hydrolyzed with 15 mL of 33% potassium hydroxide (Panreac, Barcelona, Spain), in a water bath at 100 °C during 15 min. After cooling, 0.5 mL were mixed with 50 µL of a saturated sodium sulphate solution (Merck, Darmstadt, Germany) and 2 mL of 96% ethanol (AGA, Lisbon, Portugal). Samples were placed in an ice bath for glycogen precipitation (~30 min). Following centrifugation (14,000 *g*; 8 min; 2 °C; Sigma 3K30 centrifuge, Germany) the precipitate was dissolved in 0.5 mL of distilled water, precipitated again with 1 mL of ethanol (30 min) and centrifuged (14,000 *g*; 8 min; 2 °C). The precipitate was redissolved in 0.5 mL of distilled water and 3 mL of anthrone-reagent, and the mixture was heated to 90 °C for 20 min. The absorbance was measured at 620 nm (spectrophotometer UNICAM UV-Vis, Helios, United Kingdom). A calibration curve was prepared using glycogen (Sigma, USA) as standard. The anthrone-reagent was prepared with 38 mL concentrated sulphuric acid (Fluka, Riedel-de Haën, Germany), 15 mL distilled water and 0.075 g anthrone (Merck, Darmstadt, Germany). The results were expressed as g/100g wet weight.

2.4. Chemical analyses

2.4.1. Macro, trace and toxic elements

Energy dispersive X-ray fluorescence method (EDXRF) was used to quantify the macro/trace elements S, Cl, K, Ca, Fe, Zn, Br, Cu, Se, Rb and Sr in mantle samples. The

spectrometer is a self-constructed system, using a Philips X-ray generator (PW 1140/00/60). The EDXRF technique consists of an X-ray tube equipped with a molybdenum secondary exciter. The characteristic radiations emitted by the elements in the sample were detected by a Lithium drifted Silicon [Si (Li)] detector, with 30 mm^2 active area and 8 μm beryllium window. The energy resolution was 135 eV at 5.9 keV and the acquisition system was a Nucleus PCA card. Quantitative calculations were made with the fundamental parameters method (Carvalho, Santiago, & Nunes, 2005). The X-ray generator was operated at 50 kV, 20 mA and 1000 s acquisition time. Each freeze-dried sample powder (1 g) was pressed into 2 cm diameter pellets (n = 3) without any chemical treatment and glued onto Mylar films, on sample holders, and placed directly in the X-ray beam for element determination.

Flame atomic-absorption spectrometry (FAAS; spectrometer Varian Spectr AA 55B Sydney, Australia) was used to quantify Cd and Pb in bivalve samples (n = 3) according to the procedures described by Jorhem (2000). Briefly, 10 g of mantle samples was incinerated at 500 °C and solubilized in a solution of nitric acid (15% v/v). Concentrations of Pb and Cd were calculated from linear calibration plots obtained by measurement of standard solutions absorbance prepared with $Cd(NO_3)_2$ and $Pb(NO_3)_2$ (Merck; 1 g/L dissolved in 0.5 mol/L HNO_3). A minimum of three replicate analyses was performed per sample.

Total mercury (Hg) was determined in mantle samples by atomic absorption spectrometry using an automatic Hg analyser (Leco apparatus AMA 254, St. Joseph, MI, USA) that uses the Hg vapour generation technique, according to United States Environmental Protection Agency test method 7473 (USEPA, 2007). The procedure is based on dry sample decomposition (10 mg; n = 3) by combustion, pre-concentration of Hg by amalgamation with gold and atomic absorption spectrometry. Concentrations were calculated from linear calibration plots obtained by the measurement of Hg standard solution absorbance (Merck; 1 g/L dissolved in 0.5 mol/L HNO_3).

Detection limits (DL) of each element were determined and accuracy was checked through analysis of certified biological material (Table 1): dogfish muscle (DORM-2; National Research Council of Canada), lobster hepatopancreas (TORT-2; National Research Council of Canada), oyster tissues (SRM 1566; United States National Bureau of Standards) and freeze–dried animal blood (IAEA-A-13; International Atomic Energy Agency). The concentration of all elements was reported as milligrams per kilogram on wet weight basis (mg/kg ww).

Table 1. Elemental concentration (mg/kg dw; n = 4) and detection limits (mg/kg, D.L.) of certified reference materials (average ± standard deviation) analysed by FAAS and EDXRF.

Element	Technique	D.L.	Certified value	Present work
Hg[a]	FAAS	0.005	4.64 ± 0.26	4.65 ± 0.16
Cd[b]	FAAS	0.01	26.7 ± 0.6	27.0 ± 0.1
Pb[b]	FAAS	0.02	0.35 ± 0.13	0.35 ± 0.04
As[c]	EDXRF	0.7	852 ± 14	845 ± 20
S[c]	EDXRF	10	7600*	8250 ± 400
Cl[c]	EDXRF	10	10000*	10500 ± 800
K[c]	EDXRF	10	9690 ± 50	10000 ± 60
Ca[c]	EDXRF	20	1500 ± 200	1400 ± 40
Rb[c]	EDXRF	1.1	4.45 ± 0.09	5.0 ± 0.6
Fe[a]	EDXRF	3.0	142 ± 10	148 ± 15
Cu[c]	EDXRF	0.7	63 ± 4	64 ± 2
Zn[c]	EDXRF	1.1	852 ± 14	845 ± 15
Se[c]	EDXRF	0.6	2.1 ± 0.5	2.2 ± 0.4
Br[e]	EDXRF	0.8	22 ± 3	21 ± 2
Sr[b]	EDXRF	0.5	45 ± 2	43 ± 4

[a] Dogfish muscle (DORM-2)
[b] Lobster hepatopancreas (TORT-2)
[c] Oyster tissues (SRM 1566)
[e] Freeze–dried animal blood (IAEA-A-13)
* Non-certified values provided by United States National Bureau of Standards.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used to detect significant differences in glycogen, toxic and macro/trace elements contents of the bivalves. Whenever necessary, data was transformed to satisfy normal distribution and homoscedasticity requirements. If transformed data could not meet these assumptions, non-parametric analysis of variance (Kruskall-Wallis) was performed, followed by non-parametric multiple comparison test (Dunn test). Correlations between the studied toxic and macro/trace elements were analyzed for each bivalve species with Pearson's coefficients. Differences were considered statistically significant at $p < 0.05$. All analyses were performed using the software

STATISTICA™ 7.0 (Statsoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Mortality rate and Condition Index

Table 2 shows the changes in biometric data, condition index (CI) and cumulative mortality rate of the three bivalve species analyzed during the depuration period. Mortality rate was very low in RP and MG, with no mortality in RP and only 1% mortality in MG after eight days. In contrast, SP had higher mortality rate particularly after six days (48%). Condition index (CI) was always significantly higher in RP than in MG and SP (Table 2). During depuration, CI did not show remarkable differences in RP and SP (Table 2), whereas CI significantly increased in MG during the first four days.

3.2. Glycogen content

Glycogen content varied significantly between the three species, where SP had the highest levels and MG had the lowest levels throughout the depuration period (Fig. 2). During depuration, glycogen levels significantly decreased in three bivalve species: from 1.8 to 1.4 g/100 g ww in RP; from 0.8 to 0.4 g/100 g ww in MG; and from 3.7 to 1.8 g/100 g ww in SP ($p < 0.05$; Fig. 2).

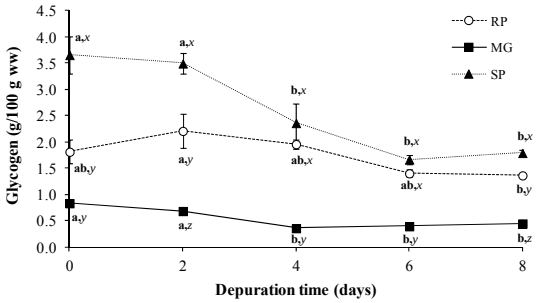


Fig. 2. Glycogen content (g/100 g, wet weight) of RP, MG and SP during the depuration time. Values represent mean ± standard deviation (n = 3). Different letters represent significant differences ($p < 0.05$) between bivalve species (x - z) or depuration time (a - b), whereas bars without letters indicate no significant differences (more statistical details in Table S1).

3.3. Elemental concentrations

The content of toxic and macro/trace elements determined in the three bivalve species during eight depuration days is shown in Fig. 3 and Table 3. Overall, all elemental concentrations varied significantly between species (Table S1). Initial concentration of Pb was highest in SP, with a mean of 1.67 mg/kg ww, while MG and RP had only 0.35 and 0.29 mg/kg ww, respectively. RP had the highest concentrations of Hg, Cd and As, with mean levels of 0.06, 0.17 and 7.3 mg/kg ww, respectively. In fact, significant higher levels were observed at day 0 for Se, Hg, Cd and As in RP, for Cl and Br in MG and for Fe, Zn, Cu,

Table 2. Biometric data (length, width, height, total weight, edible weight and total meat yield), condition index (CI) and cumulative mortality rate (%) during the depuration time of the three bivalve species (RP, MG and SP).

	Depuration (days)	Length (mm)	Width (mm)	Height (mm)	Total weight (g)	Edible weight (g)	Total meat yield (%)	CI	Mortality (%)
RP	0	50.9 ± 5.0	26.4 ± 3.4	37.3 ± 4.1	32.8 ± 11.6	14.7 ± 4.7	45.5 ± 3.2	6.3 ± 0.4 [a]	0
	2	51.5 ± 6.5	27.1 ± 3.9	38.2 ± 4.5	37.7 ± 13.8	17.1 ± 5.8	45.7 ± 2.5	6.7 ± 0.3 [a]	0
	4	47.7 ± 5.3	25.1 ± 2.8	36.9 ± 4.1	29.7 ± 9.7	13.8 ± 4.3	46.9 ± 3.5	6.6 ± 0.7 [a]	0
	6	46.5 ± 5.8	23.7 ± 4.3	34.1 ± 4.9	25.7 ± 12.5	12.0 ± 5.5	47.0 ± 2.2	6.4 ± 0.2 [a]	0
	8	46.7 ± 4.2	23.6 ± 2.8	34.3 ± 3.5	26.0 ± 7.7	11.8 ± 3.7	45.4 ± 6.0	6.5 ± 0.4 [a]	0
MG	0	57.6 ± 7.4	22.9 ± 2.1	31.7 ± 2.2	19.6 ± 4.1	9.3 ± 2.9	47.3 ± 8.0	4.6 ± 0.7 [b]	0
	2	54.4 ± 5.3	20.2 ± 2.6	29.6 ± 2.0	17.4 ± 4.8	9.3 ± 3.0	52.7 ± 5.9	5.1 ± 0.6 [ab]	0
	4	52.6 ± 5.5	22.6 ± 10.5	29.0 ± 2.4	15.7 ± 3.9	8.2 ± 2.2	52.5 ± 6.2	5.2 ± 0.2 [a]	0
	6	52.8 ± 4.4	19.3 ± 1.9	29.2 ± 2.9	16.0 ± 4.1	8.8 ± 2.4	54.9 ± 2.9	5.3 ± 0.3 [a]	0
	8	55.0 ± 4.2	20.9 ± 1.9	29.2 ± 2.6	17.6 ± 4.2	9.8 ± 2.5	55.8 ± 4.3	5.2 ± 0.4 [a]	1
SP	0	44.5 ± 3.6	13.4 ± 0.9	34.1 ± 2.4	11.2 ± 2.4	6.6 ± 1.3	59.9 ± 3.3	5.4 ± 0.2 [a]	0
	2	43.1 ± 3.2	12.5 ± 1.2	33.1 ± 2.0	9.6 ± 2.3	5.6 ± 1.6	58.4 ± 5.9	5.3 ± 0.2 [a]	3
	4	42.1 ± 3.3	12.8 ± 1.2	32.4 ± 2.2	9.8 ± 2.4	6.2 ± 1.5	63.2 ± 3.8	5.5 ± 0.3 [a]	25
	6	42.0 ± 3.7	12.6 ± 1.6	32.4 ± 2.8	9.2 ± 3.1	5.8 ± 2.2	62.0 ± 6.9	5.3 ± 0.3 [a]	48
	8	41.8 ± 3.0	12.1 ± 1.2	32.1 ± 2.3	8.4 ± 2.4	5.1 ± 1.5	60.6 ± 4.6	5.3 ± 0.1 [a]	48

Different letters represent significant differences ($p < 0.05$) in depuration time (a - b).

Table 3. Elemental composition (mean ± standard deviation; wet weight) of RP, MG and SP during the depuration time: macro elements (S, Cl, K and Ca; mg/kg, ww) and trace elements (Fe, Zn, Br Cu, Se, Rb and Sr; mg/kg, ww). Values represent mean ± SD (n = 3).

		Depuration time (days)				
Parameter	**Species**	**0**	**2**	**4**	**6**	**8**
S	RP	2807 ± 258^{a,x}	3148 ± 356^{a,x}	3425 ± 192^{a,x}	3099 ± 443^{a,x}	3602 ± 330^{a,x}
	MG	2836 ± 64^{b,x}	3164 ± 131^{ab,x}	3325 ± 140^{b,x}	3231 ± 349^{ab,x}	3557 ± 102^{a,x}
	SP	1804 ± 26^{a,y}	1737 ± 130^{a,y}	1695 ± 211^{a,y}	1852 ± 521^{a,x}	1799 ± 41^{a,y}
Cl	RP	6603 ± 1338^{b,y}	9781 ± 502^{a,y}	8154 ± 756^{ab,xy}	7638 ± 623^{ab,y}	7698 ± 1364^{ab,x}
	MG	11642 ± 256^{b,x}	14316 ± 392^{a,x}	10409 ± 202^{b,x}	10864 ± 1059^{b,x}	10103 ± 132^{b,x}
	SP	5462 ± 290^{c,y}	8358 ± 375^{a,y}	6805 ± 613^{b,y}	7944 ± 381^{ab,xy}	7031 ± 33^{ab,x}
K	RP	1819 ± 177^{a,x}	1959 ± 38^{a,x}	2077 ± 91^{a,x}	2080 ± 64^{a,x}	2056 ± 19^{a,x}
	MG	1661 ± 96^{a,x}	1723 ± 84^{a,x}	1725 ± 89^{a,y}	1637 ± 295^{a,x}	1662 ± 133^{a,y}
	SP	2229 ± 197^{a,x}	2202 ± 176^{a,x}	2184 ± 12^{a,x}	2064 ± 385^{a,x}	1925 ± 21^{a,xy}
Ca	RP	522 ± 96^{a,x}	411 ± 79^{a,x}	608 ± 256^{a,x}	788 ± 145^{a,xy}	692 ± 62^{a,x}
	MG	491 ± 59^{a,x}	456 ± 53^{a,x}	475 ± 74^{a,x}	379 ± 98^{a,y}	461 ± 161^{a,x}
	SP	686 ± 70^{a,x}	626 ± 98^{a,x}	588 ± 163^{a,x}	874 ± 77^{a,x}	720 ± 11^{a,x}
Fe	RP	184 ± 56^{a,y}	58 ± 2^{b,y}	67 ± 18^{b,y}	55 ± 3^{b,x}	88 ± 31^{b,y}
	MG	97 ± 4^{a,y}	36 ± 6^{b,y}	31 ± 0^{b,y}	26 ± 6^{b,y}	32 ± 7^{b,y}
	SP	700 ± 4^{a,x}	303 ± 10^{b,x}	306 ± 63^{b,x}	429 ± 0^{b,y}	241 ± 0^{b,x}
Zn	RP	15 ± 2^{a,z}	16 ± 2^{a,y}	16 ± 2^{a,z}	16 ± 1^{a,z}	17 ± 1^{a,y}
	MG	39 ± 4^{b,y}	50 ± 4^{a,y}	58 ± 13^{a,y}	57 ± 4^{a,y}	57 ± 18^{a,y}
	SP	125 ± 2^{b,x}	157 ± 18^{b,x}	138 ± 7^{b,x}	180 ± 0^{ab,x}	204 ± 0^{a,x}
Br	RP	51 ± 1^{a,y}	53 ± 1^{a,y}	38 ± 5^{b,y}	37 ± 1^{b,y}	40 ± 2^{b,y}
	MG	142 ± 2^{a,x}	112 ± 4^{b,x}	80 ± 4^{c,x}	82 ± 2^{c,x}	86 ± 0^{c,x}
	SP	25 ± 2^{b,z}	38 ± 2^{a,z}	32 ± 2^{ab,y}	39 ± 4^{a,y}	40 ± 0^{a,y}
Cu	RP	3.1 ± 0.6^{a,xy}	3.1 ± 0.8^{ab,x}	2.1 ± 0.4^{ab,x}	2.0 ± 0.0^{b,y}	2.2 ± 0.1^{ab,y}
	MG	1.7 ± 0.1^{a,y}	1.3 ± 0.2^{a,x}	1.6 ± 0.1^{a,x}	1.5 ± 0.1^{a,z}	1.6 ± 0.1^{a,z}
	SP	3.5 ± 0.3^{a,x}	2.8 ± 0.3^{ab,x}	2.5 ± 0.2^{b,x}	3.3 ± 0.0^{ab,x}	3.5 ± 0.0^{a,x}
Se	RP	1.6 ± 0.2^{a,x}	2.1 ± 0.6^{a,x}	1.3 ± 0.2^{a,x}	1.2 ± 0.0^{a,x}	1.5 ± 0.2^{a,x}
	MG	0.5 ± 0.1^{a,y}	0.6 ± 0.0^{a,y}	0.6 ± 0.1^{a,y}	0.6 ± 0.0^{a,y}	0.6 ± 0.1^{a,y}
	SP	0.4 ± 0.0^{a,y}	0.4 ± 0.1^{a,y}	0.4 ± 0.0^{a,y}	0.4 ± 0.0^{a,z}	0.5 ± 0.0^{a,y}
Rb	RP	2.0 ± 0.0^{a,y}	0.8 ± 0.2^{b,x}	0.7 ± 0.1^{b,xy}	0.7 ± 0.2^{b,x}	0.6 ± 0.0^{b,x}
	MG	0.8 ± 0.3^{a,z}	0.4 ± 0.0^{a,x}	0.5 ± 0.2^{a,y}	0.8 ± 0.4^{a,x}	0.6 ± 0.5^{a,x}
	SP	3.0 ± 0.2^{a,x}	1.0 ± 0.1^{b,x}	1.0 ± 0.0^{b,x}	1.0 ± 0.3^{b,x}	1.0 ± 0.0^{b,x}
Sr	RP	7.0 ± 0.0^{ab,y}	7.6 ± 0.5^{ab,y}	7.3 ± 0.0^{b,y}	9.2 ± 0.3^{a,y}	7.4 ± 0.0^{ab,z}
	MG	11.7 ± 0.0^{a,x}	11.2 ± 0.3^{a,x}	7.1 ± 0.7^{b,y}	8.0 ± 1.6^{b,y}	10.2 ± 0.0^{b,y}
	SP	12.2 ± 0.3^{b,x}	11.8 ± 0.0^{b,x}	11.3 ± 0.9^{b,x}	17.2 ± 2.0^{a,x}	17.6 ± 0.0^{a,x}

For each element, different letters represent significant differences ($p < 0.05$) between bivalve species (x - z) or depuration time (a - d).

Rb and Pb in SP, whereas no differences were detected for K and Ca. Overall, such differences between species were maintained during depuration, except in Rb. In addition, S concentration was always identical between RP and MG, but significantly higher than in SP. And Sr levels were similar between MG and SP, but significantly higher than in RP after two depuration days, when these levels were higher only in SP.

During depuration time, significant changes were observed for Cl, Fe, Zn, Br, Rb, Hg, Pb, Sr, Cd, Cu, S and As levels, but no changes were found in the concentration of K, Ca and Se (Tables 3 and 4; Supplemental Table S1). The elements reduction/increase during depuration of the different bivalve species is presented in Table 4. In RP, significant reductions were detected for Fe, Br, Cu, Rb, Hg, Cd, Pb and As. Mercury, Cd and As showed the slowest depuration rates, with only 32, 38 and 19%, respectively, after six-eight days. On the other hand, Fe, Rb and Pb showed the fastest depuration rates in this

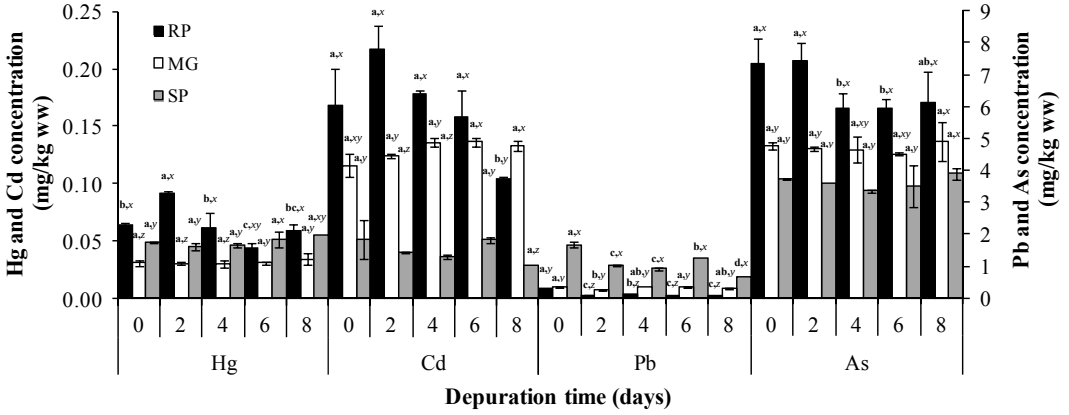


Fig. 3. Toxic elements concentration (Hg, Cd, Pb and As; mg/kg wet weight) of RP, MG and SP during depuration time. Values represent mean ± standard deviation (n = 3).

species, with 68, 61 and 71%, respectively, after only two days. In contrast, a significant increase was observed for Cl and Sr in this species, being a faster accumulation rate found in Cl (48% after two days) and a slower rate in Sr (32% after six days). In MG, significant reductions were observed for Cl, Fe, Br, Sr and Pb. Chlorine and Sr showed the slowest depuration rates, with only 11 and 39%, respectively, after four days, whereas Fe, Br and Pb revealed the fastest depuration rates, with 73, 21 and 27%, respectively, after only two days. However, a significant increase was detected in this species for S and Zn, being registered a slow accumulation rate in both elements (17 and 48%, respectively, after four days). Finally, in SP the elements that showed a significant reduction throughout the depuration period were Fe, Cu, Rb, Pb and As. Copper and As showed the slowest depuration rates, with only 28 and 10%, respectively, after four days in this species, whereas Fe, Rb and Pb revealed the fastest depuration rates, with 57, 66 and 39%, respectively, after only two days. In contrast, this species revealed an increase in levels of Cl, Zn, Br and Sr, being a faster accumulation rate found in Cl and Br (53% in both, after two days) and a slower rate in Zn and Sr (45 and 41%, respectively, after six days).

Additionally, the results of Pearson correlation analysis (Table S2) revealed that Rb and Pb were positively correlated in the three species. In RP and SP, iron also showed a significant positive linear relationship with Rb and Pb, and Hg was positively correlated with As. Other statistically positive correlations were found in RP (e.g. S-Zn, Br-Cu, Br-Se, Br-Hg, Br-As, Cu-Se, Cu-Hg, Cu-As, Se-Hg and Se-As), MG (e.g. Ca-As, Fe-Br and Zn-Cd) and SP (e.g. S-As, Cl-Br, Ca-Sr, Zn-As, Br-Sr and Cd-Pb), as well as significant negative linear relationships (in RP: K-Rb, K-Pb and K-As, Ca-Br, Ca-Hg, Ca-Cd and Ca-As; in MG: S-Fe, S-Br, Cl-Pb, K-Rb, Fe-Cd and Br-Cd; and SP: Cl-Fe, Cl-Rb, K-Sr, Fe-Br and Br-Rb). Concerning the correlations between element concentrations and CI or glycogen content, significant relationships were only found in RP and MG. In fact, CI was positively correlated with S in RP, and with Zn and Cd in MG, while glycogen showed positive correlations with Fe (MG), Br (RP and MG), Hg (RP) and Cd (RP), and negative linear relationships with Ca (RP), S (MG) and Cd (MG).

4. Discussion

Overall, the results obtained show that the initial levels of toxic elements (Hg, Pb and Cd), were well below the MPLs (EC, 2006) in all studied bivalve species, except Pb in SP which exceeded the legal value (1.67 mg/kg), whereby this species was currently declared unfit for human consumption (DR, 2013). Caçador et al. (2012) found higher levels of Pb (31.8 μg/g dw corresponding roughly to 6.0 μg/g ww) and even Cd (2.6 μg/g dw corresponding to 0.5 μg/g ww) for this species in other locations of Tagus estuary.

Table 4. Effect of depuration time on change in concentration (% reduction/increase) of macro (S, Cl, K and Ca), trace (Fe, Zn, Br, Cu, Se, Rb and Sr) and toxic elements (Hg, Cd, Pb and As) of RP, MG and SP.

		Depuration time (days)			
Parameter	**Species**	**0–2**	**0–4**	**0–6**	**0–8**
S	RP	+12.2	+22.0	+10.4	+28.3
	MG	+11.6	**+17.2***	+13.9	**+25.4***
	SP	-3.7	-6.1	+2.6	-0.3
Cl	RP	**+48.1***	+23.5	+15.7	+16.6
	MG	**+23.0***	**-10.6***	-6.7	**-13.2***
	SP	**+53.0***	**+24.6***	**+45.4***	**+28.7***
K	RP	+7.7	+14.2	+14.3	+13.0
	MG	+3.7	+3.9	-1.5	+0.1
	SP	+1.2	+2.0	+7.4	+13.6
Ca	RP	-21.2	+16.4	+51.0	+32.4
	MG	-7.2	-3.3	-22.7	-6.1
	SP	-8.8	-14.3	+27.4	+4.9
Fe	RP	**-68.4***	**-63.6***	**-70.3***	**-52.0***
	MG	**-62.7***	**-68.3***	**-72.8***	**-67.2***
	SP	**-56.7***	**-56.2***	**-38.6***	**-65.6***
Zn	RP	+4.3	+6.4	+4.3	+12.8
	MG	+26.6	**+48.0***	**+45.8***	**+44.0***
	SP	+25.9	+11.0	**+44.8***	**+63.6***
Br	RP	+3.8	**-24.9***	**-27.4***	**-22.1***
	MG	**-21.4***	**-43.9***	**-42.2***	**-39.4***
	SP	**+52.8***	**+28.9***	**+55.6***	**+58.0***
Cu	RP	-1.6	**-32.6***	**-36.5***	**-28.7***
	MG	-23.7	-6.4	-12.2	-6.0
	SP	-19.5	**-27.9***	-7.7	-2.1
Se	RP	+32.2	-17.4	-23.7	-5.1
	MG	+4.3	+12.5	+10.1	+14.2
	SP	+3.1	+9.8	-2.6	+21.4
Rb	RP	**-61.3***	**-67.7***	**-63.9***	**-68.3***
	MG	-49.9	-33.3	+7.1	-21.0
	SP	**-65.8***	**-66.4***	**-66.2***	**-66.6***
Sr	RP	+8.2	+3.6	**+31.6***	**+5.3***
	MG	-4.5	**-39.1***	**-31.9***	**-12.6***
	SP	-3.3	-7.2	**+40.9***	**+44.0***
Hg	RP	**+42.5***	-4.6	**-32.2***	-8.1
	MG	-2.7	-2.8	-0.7	+10.1
	SP	-7.9	-5.6	+4.2	+13.9
Cd	RP	+29.0	+5.8	-6.3	**-37.8***
	MG	+7.2	+17.3	+17.4	+14.4
	SP	-21.1	-29.7	-0.4	-42.4
Pb	RP	**-70.5***	**-52.9***	**-69.2***	**-67.9***
	MG	**-26.6***	-6.2	+0.8	-10.5
	SP	**-38.8***	**-45.1***	**-24.3***	**-60.0***
As	RP	+1.4	**-19.0***	**-19.2***	-16.4
	MG	-1.6	-2.6	-5.5	+2.9
	SP	-3.5	**-10.1***	-5.9	+4.0

The signal (-) indicated a reduction and (+) an increase. An asterisk (*) indicates a statistically significant differences.

Freitas *et al.* (2012) found higher levels for As, Cd and Hg as well as for Cu, Zn, in RP from Ria de Aveiro (northwest Atlantic coast of Portugal) but similar levels for Pb. Regarding As, no legal limits in European Union for seafood have been established so far. Nevertheless, the Food and Drug Administration (FDA, 1993) has set maximum allowable levels for total As at 86 mg/kg for bivalves, which was not exceeded by all studied species. It is known that arsenic can be found in various chemical forms and oxidation states in seafood that differ in their degree of toxicity and the pathologies associated with them (Muñoz *et al.*, 2000). As a general rule, inorganic arsenic, which predominates in seawater and sediments, is the most toxic form which can damage DNA and cause cancer. However, most of the arsenic in seafood is available in non-toxic organic forms, such as arsenobetaine (Lai, Cullen, & Ray, 1999). The current study revealed significantly higher levels of toxic elements in RP (Hg, Cd and As) and SP (Pb) compared to MG, which could be probably due to the differences in biological characteristics (or habitat) of the three species. Indeed, clam species usually live burrowed in the sediment at a depth ranging from 3–4 cm (in RP) to 5–20 cm (in SP), acting both as deposit and suspension feeders (Akberali, & Davenport, 1981; Hughes, 1970; Jones, Sanford, & Jones, 1993; Kondo, 1987). Mussels such as MG are generally attached by byssal threads to intertidal rocky shores (FAO, 2014), and are only exposed to metal uptake from the water at high tide, not from the sediments. Toxicokinetic studies conducted by Labrot, Narbonne, Ville, Saint Denis, and Ribera (1999) have also found that the rate and level of accumulation of toxic elements depend on the species and element. Moreover, the concentrations of toxic elements were probably higher in the sediment where both clam species were collected near the industrial areas of Lisbon (Barreiro and Vila Franca de Xira), that are responsible for most of the contamination of estuarine sediments in this area.

Overall, the depuration of the different bivalve species collected in Tagus estuary

was effective for some elements but also dependent on duration of the process. Several authors (Anandraj, Marshall, Gregory, & McClurg, 2002; Barsyte-Lovejoy, 1999; Khayatzadeh, & Abbasi, 2010) reported that bivalve molluscs have a depuration mechanism to reduce accumulation of toxic elements in their body through the excretion of metabolites. However, depuration of accumulated toxic elements has been reported as a slow process, since low efficiency was registered for Cd and Pb in three clam species during seven depuration days (Cheung, & Wong, 1997). Additionally, the effectiveness of the depuration process is also dependent on a number of variables including the health status of the bivalves, environmental parameters within the depuration plant (e.g. temperature, salinity), the type of metal and level of contamination (Jackson, & Ogburn, 1999). Overall, the three species of bivalves studied displayed different depuration rates likely due to their different filtration and excretion rates, affinity in elemental uptake and element metabolization process. The reduction of Pb, particularly in SP after two depuration days is of great importance for suppliers and retailers since the levels were well below the MPLs after this short depuration period. In this way, SP could be potentially consumed after only two days of depuration as far as regulated toxic elements are concerned. On the other hand, other elements (non-toxic) significantly increased their concentration throughout the depuration period, which is likely due to higher levels of these elements in seawater from the depuration facilities. However, the accumulation pattern varied according to species thus reflecting distinct metabolic mechanisms. The elements K, Ca and Se were neither depurated or accumulated throughout the experiment in any of the three species.

Roesijadi (1981) related the high affinities of elements to bind with metallothioneins to their ability to fix themselves within the different tissues. These metallothioneins are non-enzymatic proteins involved in the sequestration and detoxification of excessive elements, acting as a protective mechanism against the elemental toxicity through the prevention of interactions with critical cellular components such as enzymes, structural proteins, DNA and membrane lipids (Ivanina, Cherkasov, & Sokolova, 2008). Examples of high-affinity binding of toxic elements are Cd (Amiard, Amiard-Triquet, Barka, Pellerin, & Rainbow, 2006) and Hg (Purina, Barda, Rimsa, Poikane, & Jansons, 2013), and essential elements as Cu (Amiard *et al.*, 2006) and Zn (Palmiter, 2004). Contrarily, Ruddell (1971) and El-Gamal (2011) explained that high depuration of elements from edible tissues reflect low affinities and the elements that can be eliminated may be bound in amoebocytes (amoeboid lymphocytes) and not fixed in bivalve tissues. Therefore, the high depuration of Pb and Fe probably reflects its weak binding to bivalve tissues. Previous studies reported higher Fe, Cu, Zn and Cd reductions in other bivalve species (*Paphia undulata*, *Ruditapes decussatus*, *Crassostrea gigas* and *Mytilus smarangdium*) subjected to depuration compared to the present study (El-Gamal, 2011; El-Shenawy, 2004; Gnassia-Barelli, Roméo, & Puiseux-Daob, 1995; Han, Jeng, Tsai, & Jeng, 1993). Yet, lower Pb and Zn reduction were highlighted for *P. undulata* and *M. smarangdium* (El-Gamal, 2011; Han *et al.*, 1993). These results clearly indicate that the pattern and efficiency of depuration rates of toxic and macro/trace elements are influenced by species, time of depuration, element, initial elemental levels in bivalves and concentration of elements in the water of the depuration facility. Interestingly, results of Pearson correlation analysis revealed significantly positive correlations between elements contents in the three bivalve species during depuration, particularly between Pb and Rb, indicating that several elements may have similar affinity or metabolization processes. In contrast, negative correlations were also detected between some macro/trace and toxic elements in bivalve species during depuration (e.g. K-Pb, K-As, Ca-Hg, Ca-Cd, Ca-As, Cl-Pb, Fe-Cd and Br-Cd) which may as well indicate that a competition might occur between these elements in bivalve metabolism. According to Suter (2007), certain non-essential metals may share

binding sites with essential metals. For instance, calcium can inhibit the uptake of several toxic metals such as cadmium by bivalve species through Ca channel blockers (Qiu, Xie, & Wang, 2005; Roesijadi, & Unger 1993; Wang, & Evans, 1993). Fan, Wang, and Chen (2002) found a decrease of Cd assimilation efficiencies with increasing Zn concentrations in two marine bivalves (*Perna viridis* and RP). Inverse relationships have also been reported between Zn and Pb implying that bivalves actively take up Zn from the surrounding water in an attempt to restrict the accumulation of toxic metals in the interstitial tissues (Jana, & Das, 1997). Generally, bivalves are organisms with great ability to regulate the essential metal concentration and detoxify non-essential metals (Thorsen, Cope, & Shea, 2007). Additionally, the correlations varied widely according to bivalve species indicating different elemental metabolization processes, with RP revealing more significant correlations between elements, which may indicate a greater interaction between the elements in this species during depuration.

Purina *et al.* (2013) referred that concentrations of Hg and also metallothioneins in bivalves were related to their condition index, suggesting that the accumulation of toxic elements in these organisms depends on their physiological status; probably healthier animals are more likely to accumulate toxic elements. CI was positively correlated with S (in RP), Zn (in MG) and Cd (in MG) in this study, indicating a possible effect of the physiological status of these species. In this sense, the species in good physiological conditions have a higher filtration rate which means a more uptake of the elements from the food organisms or directly from the filtered water. Furthermore, the high mortality in SP and reduced glycogen content may be due to the stressful conditions (e.g. lack of feed and sediment) during the depuration process, leading to a greater utilization of biochemical reserves. Indeed, under stressful depuration conditions, glycogen levels are usually used for maintenance needs and reflect bivalve capacity to sustain further stress (Patrick, Faury, & Goulletquer, 2006). The reduction or depletion of glycogen reserves is often associated with mass mortalities of bivalve species (Patrick *et al.*, 2006; Uzaki, Kai, Aoyama, & Suzuki, 2003). This indicates that SP should not be depurated for more than two days, which was enough to reduce the Pb level of this species significantly.

5. Conclusion

The results obtained in this study clearly indicate that levels of toxic, macro and trace elements vary between species and that they are affected by the duration of the depuration process. The depuration was effective to reduce the levels of toxic elements like Pb, particularly in SP to permissible levels after only two days. This clearly indicates that depuration may be an excellent mitigation strategy to reduce Pb levels in contaminated bivalves to levels considered as acceptable for human consumption. However, for SP depuration for more than two days resulted in high mortality rates. In general, Fe, Cu, Br and Rb levels were also significantly reduced during depuration, whereas Cl, Zn and Sr showed a significant increase. In contrast, K, Ca and Se levels were not affected by depuration in the three bivalve species. RP and MG did not show any mortality during the eight days of depuration. Therefore, SP should be only depurated for a maximum period of two days. Glycogen content was a suitable biomarker to assess the health status of bivalve species during depuration. Overall, this study is particularly useful to increase the awareness of seafood safety authorities and stakeholders of the benefits of depuration as a mitigation tool to decrease toxic chemicals levels (e.g. lead) in bivalves.

Acknowledgments

The first author acknowledges a PhD scholarship of the Portuguese Foundation for Science and Technology (FCT; Ref. SFRH/BD/69274/2010), whereas the last two authors were supported by a research contract from the IF 2013 and Ciência 2008 Programmes from FCT.

References

Akberali, H. B., & Davenport, J. (1981). The responses of the bivalve *Scrobicularia plana* (da Costa) to gradual salinity changes. *Journal of Experimental Marine Biology and Ecology, 53,* 251–259.

Akberali, H. B., & Trueman, E. R. (1985). Effects of environmental stress on marine bivalve molluscs. In J. H. S. Blaxter, F. S. Russell, & M. Yonge (Eds.), *Advances in marine biology.* (Volume 22). London: Academic Press. p. 101–197.

Almeida, C., & Soares, F. (2012). Microbiological monitoring of bivalves from the Ria Formosa Lagoon (south coast of Portugal): a 20 years of sanitary survey. *Marine Pollution Bulletin, 64,* 252–262.

Amiard, J.-C., Amiard-Triquet, C., Barka, S., Pellerin, J., & Rainbow, P. S. (2006). Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquatic Toxicology, 76,* 160–202.

Anacleto, P., Barrento, S., Nunes, M. L., Rosa, R., & Marques, A. (2014). Portuguese consumers' attitudes and perceptions of bivalve molluscs. *Food Control, 41,* 168–177.

Anandraj, A., Marshall, D. J., Gregory, M. A., & McClurg, T. P. (2002). Metal accumulation, filtration and O2 uptake rates in the mussel *Perna perna* (Mollusca: Bivalvia) exposed to Hg^{2+}, Cu^{2+}, and Zn^{2+}. *Comparative Biochemistry and Physiology – Part C: Toxicology & Pharmacology, 132,* 355–363.

Baird, R. H. (1958). Measurement of condition in mussels and oysters. *Journal du Conseil Permanent International pour l'Exploration de la Mer, 23,* 249–257.

Barber, B. J., & Blake, N. J. (1981). Energy storage and utilization in relation to gametogenesis in *Argopecten irradians concentricus* (Say). *Journal of Experimental Marine Biology and Ecology 52,* 121–134.

Barile, N. B., Scopa, M., Nerone, E., Mascilongo, G., Recchi, S., Cappabianca, S., *et al.* (2009). Study of the efficacy of closed cycle depuration system on bivalve molluscs. *Veterinaria Italiana, 45(4),* 555–566.

Barsyte-Lovejoy, D. (1999). Heavy metal concentrations in water, sediment and mollusc tissues. *Acta Zoologica Lituanica Hydrobiologia, 9(2),* 12–20.

Bressan, M., & Marin, M. G. (1985). Seasonal variations in biochemical composition and condition index of cultured mussels (*Mytillus galloprovincialis*) in the Lagoon of Venice (North Adriatic). *Aquaculture, 48,* 13–21.

Brogueira, M. J., & Cabeçadas, G. (2006). Identification of similar environmental areas in Tagus estuary by using multivariate analysis. *Ecological Indicators, 6,* 508–515.

Caçador, I., Costa, J. L., Duarte, B., Silva, G., Medeiros, J. P., Azeda, C., *et al.* (2012). Macroinvertebrates and fishes as biomonitors of heavy metal concentration in the Seixal Bay Tagus estuary: which species perform better?. *Ecological Indicators, 19,* 184–190.

Carvalho, M. L., Santiago, S., & Nunes, M. L. (2005). Assessment of the essential element and heavy metal content of edible fish muscle. *Analytical and Bioanalytical Chemistry, 382(2),* 426–432.

Censi, P., Spoto, S. E., Saiano, F., Sprovieri, M., Mazzola, S., Nardone, G., *et al.* (2006). Heavy metals in coastal water systems. A case study from the northwestern Gulf of Thailand. *Chemosphere, 64,* 1167–1176.

Cheung, Y. H., & Wong, M. H. (1997). Depuration and bioaccumulation of heavy metals by clams from tolo harbour, Hong Kong. *Toxicological & Environmental Chemistry, 58(1-4),* 103–116.

Cozzi, L., Suffredini, E., Ciccaglioni, G., & Croci, L. (2009). *Depuration treatment of mussels experimentally contaminated with V. parahaemolyticus and V. vulnificus.* Nantes, France: Proceeding ICMSS09. p. 6.

Dallinger, R. (1995). Metabolism and toxicity of metals: metallothioneins and metal elimination. In M. P. Cajaraville (Ed.), *Cell biology in environment toxicology* (pp. 171–190). Bilbao: Universidade del Pais Vasco.

DR (Diário da República). (2013). *Despacho n.º 15264/2013 do Diário da República, 2.ª série, N.º 227, de 22 de Novembro de 2013.* p. 34255–34258.

EC (Council Regulation). (2004a). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for food of animal origin. *Official Journal of the European Union, L226,* 22–82.

EC (Council Regulation). (2004b). Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal of the European Union, L226,* 83–127.

EC (Council Regulation). (2006). Commission Regulation (EC) No 1881/2006 of 19 De-cember 2006. Setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union, L364,* 5–24.

El-Gamal, M. M. (2011). The effect of depuration on heavy metals, petroleum hydrocarbons, and microbial contamination levels in *Paphia undulata* (Bivalvia: Veneridae). *Czech Journal of Animal Science, 56(8),* 345–354.

El-Shenawy, N. S. (2004). Heavy metal and microbial depuration of the clam *Ruditapes decussatus* and its effect on bivalve behavior and physiology. *Environmental Toxicology, 19,* 143–153.

Emmanouil, C., Kypriotakis, S., Kungolos, A., & Machera, K. (2008). Effects of Thiram or MCPA acid on mussel gill DNA. *Discussion Paper Series, 14(24),* 455–468.

EUROSTAT (2014). *Statistics – Fisheries database.* 07 July 2014. Available at http://epp.eurostat.ec.europa.eu/portal/page/portal/eurostat/home/.

Fan, W., Wang, W. X., & Chen, J. (2002). Geochemistry of Cd, Cr, and Zn in highly contaminated sediments and its influences on assimilation by marine bivalves. *Environmental Science and Technology, 36,* 5164–5171.

Fang, Z. Q., Cheung, R., & Wong, M. H. (2003). Heavy metals in oysters, mussels and clams collected from coastal sites along the Pearl River Delta, South China. *Journal of Environmental Sciences (China), 15,* 9–24.

FAO (Food and Agriculture Organization). (2014). *Species Fact Sheets: Mytilus galloprovincialis (Lamark, 1819).* 17 March 2014. Available at http://www.fao.org/fishery/culturedspecies/Mytilus_galloprovincialis/en.FDA (Food and Drug Administration). (1993). *Guidance document for arsenic in shellfish.* Washington, DC: Food and Drug Administration, U.S. Department of Health and Human Services, Public Health Service, Office of Seafood. p. 44.

Fernandez-Castro N, & De Vido de Mattio N. (1987). Biochemical composition index and energy value of *Ostrea puelchanan* (D'Orbigny): relationships with the reproductive cycle. *Journal of Experimental Marine Biology and Ecology, 108,* 113–126.

Fernández-Tajes, J., Flórez, F., Pereira, S., Rábade, T., Laffon, B., & Méndez, J. (2011). Use of three bivalve species for biomonitoring a polluted estuarine environment. *Environmental Monitoring and Assessment, 177,* 289–300.

Förstner, U., & Wittmann, G. T. W. (1979). *Metal pollution in the aquatic environment.* Berlin: Springer-Verlag. p. 486.

Freitas, R., Pinto, L. R., Sampaio, M., Costa, A., Silva, M., Rodrigues, A. M., *et al.* (2012). Effects of depuration on the element concentration in bivalves: comparison between sympatric *Ruditapes decussatus* and *Ruditapes philippinarum. Estuarine, Coastal and Shelf Science, 110,* 43–53.

Gnassia-Barelli, M., Roméo, M., & Puiseux-Daob, S. (1995). Effects of cadmium and copper contamination on calcium content of the bivalve *Ruditapes decussatus*. *Marine Environmental Research, 39(1–4),* 325–328.

Gupta, S. K., & Singh, J. (2011). Evaluation of mollusc as sensitive indicator of heavy metal pollution in aquatic system: a review. *IIOAB Journal, 2,* 49–57.

Han, B.-C., Jeng, W.-L., Tsai, Y.-N., & Jeng, M.-S. (1993). Depuration of copper and zinc by green oysters and blue mussels of Taiwan. *Environmental Pollution, 82,* 93–97.

Hughes, R. N. (1970). Population dynamics of bivalve *Scrobicularia plana* (Da Costa) on an intertidal mud-flat in North Wales. *Journal of Animal Ecology, 39,* 333–356.

Ivanina, A. V., Cherkasov, A. S., & Sokolova, I. M. (2008). Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. *Journal of Experimental Biology, 211,* 577–586.

Jackson, K. L., & Ogburn, D. M. (1999). *Review of depuration and its role in shellfish quality assurance* (FRDC Project No. 96/355). Pyrmont, Australia: NSW Fisheries Final Report Series No. 13. p. 77.

Jana, B. B., & Das, S. (1997). Potential of freshwater mussel (*Lamellidens marginalis*) for cadmium clearance in a model system. *Ecological Engineering, 8,* 179–193.

Jones, G. G., Sanford, C. L., & Jones, B. L. (1993). *Manila clams: hatchery and nursery methods.* Victoria, BC: Innovative Aquaculture Products Ltd. and Science Council of British Columbia. p. 73.

Jorhem, L. (2000). Determination of metals in foods by atomic absorption spectrometry after dry ashing: NMKL collaborative study. *Journal of AOAC International, 83,* 1204–1211.

Khayatzadeh, J., & Abbasi, E. (2010). *The effects of heavy metals on aquatic animals.* Iran: The 1st International Applied Geological Congress, Department of Geology, Islamic Azad University - Mashad Branch, 26-28 April. p. 688–694.

Kondo, Y. (1987). Burrowing depth of infaunal bivalves – observation of living species and its relation to shell morphology. *Transactions and Proceedings of the Palaeontological Society of Japan (New Series), 148,* 306–323.

Labrot, F., Narbonne, J. F., Ville, P., Saint Denis, M., & Ribera, D. (1999). Acute toxicity, toxicokinetics, and tissue target of lead and uranium in the clam *Corbicula fluminea* and the worm *Eisenia fetida*: comparison with the fish *Brachydanio rerio*. *Archives of Environmental Contamination and Toxicology, 36,* 167–178.

Laffon, B., Rábade, T., Pásaro, E., & Méndez, J. (2006). Monitoring of the impact of Prestige oil spill on *Mytilus galloprovincialis* from Galician coast. *Environment International, 32,* 342–348.

Lai, V. W.-M, Cullen, W. R., & Ray, S. (1999). Arsenic speciation in scallops. *Marine Chemistry, 66(1),* 81–89.

Lee, R., Lovatelli, A., & Ababouch, L. (2008). *Bivalve depuration: fundamental and practical aspects.* Rome: FAO Fisheries Technical Paper. No. 511, FAO. p. 161.

Lee, R. J., & Younger, A. D. (2002). Developing microbiological risk assessment for shellfish purification. *International Biodeterioration and Biodegradation, 50,* 177–183.

Lees, D., Younger, A., & Dore, B. (2010). Depuration and relaying. In G. Rees, K. Pond, D. Kay, J. Bartram, & J. Santo Domingo (Eds.), *Safe management of shellfish and harvest waters* (pp. 145–181). London: World Health Organization (WHO), IWA Publishing.

Lemiere, S., Cossu-Leguille, C., Bispo, A., Jourdain, M. J., Lanhers, M. C., Burnel, D., *et al.* (2005). DNA damage measured by the single-cell gel electrophoresis (comet) assay in mammals fed with mussels contaminated by the 'Erika' oil-spill. *Mutation Research, 581,* 11–21.

Leyva, A., Quintana, A., Sánchez, M., Rodríguez, E. N., Cremata, J., & Sánchez, J. C. (2008). Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: method development and validation. *Biologicals,* 36, 134–141.

Livingstone, D. R. (1993). Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *Journal of Chemical Technology and Biotechnology, 57,* 195–211.

Maguire, J. A., Fleury, P. G., & Burnell, G. M. (1999). Some methods for quantifying quality in the scallop *Pecten maximus* (L.*). Journal of Shellfish Research, 18,* 59–66.

Manfra, L., & Accornero, A. (2005). Trace metal concentrations in costal marine waters of the center Mediterranean. *Marine Pollution Bulletin, 50,* 686–962.

Mann, R. (1978). A comparison of morphometric, biochemical and physiological indexes of condition in marine bivalve molluscs. In: J. H. Thorpe, & J. W. Gibbons (Eds.), *Energy and environmental stress in aquatic system* (pp. 484–497). Oak Ridge, TN: Technical Information Center, U.S. Department of Energy.

Muñoz, O., Devesa, V., Suñer, M. A., Vélez, D., Montoro, R., Urieta, I., *et al.* (2000). Total and inorganic arsenic in fresh and processed fish products. *Journal of Agricultural and Food Chemistry, 48(9),* 4369–4376.

Palmiter, R. D. (2004). Protection against zinc toxicity by metallothionein and zinc transporter 1. *Proceedings of the National Academy of Sciences, 101(14),* 4918–4923.

Patrick, S., Faury, N., & Goulletquer, P. (2006). Seasonal changes in carbohydrate metabolism and its relationship with summer mortality of Pacific oyster *Crassostrea gigas* (Thunberg) in Marennes-Oleron bay (France). *Aquaculture, 252,* 328–338.

Phillips, D. J. H. (1980). *Quantitative aquatic biological indicators: their use monitor trace metal and organochlorine pollution.* London: Applied Science Publishers Ltd. p. 488.

Purina, I., Barda, I., Rimsa, E., Poikane, R., & Jansons, M. (2013). Concentrations of metallothionein in the bivalve molluscs *Anodonta* spp. and Unio spp. from Latvian lakes with different anthropogenic pressure. *E3S Web of Conferences, 1,* 34005.

Qiu, J.-W., Xie, Z.-C., & Wang, W.-X. (2005). Effects of calcium on the uptake and elimination of cadmium and zinc in Asiatic clams. *Archives of Environmental Contamination and Toxicology, 48,* 278–287.

Raposo, J. C., Bartolome, E. L., Cortazar, E. E., Arana, E. J., Zabaljauregui, E. M., de Diego, E. M., *et al.* (2009). Trace metals in oysters, *Crassotrea* spp., from UNESCO protected natural reserve of Urdaibai: space-time observations and source identification. *Bulletin of Environmental Contamination and Toxicology, 83,* 223–229.

RASFF (Rapid Alert System for Food and Feed). (2012). *The Rapid Alert System for Food and Feed (RASFF) – Annual report 2011.* Luxembourg: Publications Office of the European Union. p. 47.

Roesijadi, G. (1981). The significance of low molecular weight, metallothionein-like protein in marine invertebrates: current status. *Marine Environmental Research, 4(3),* 167–179.

Roesijadi, G., & Unger, M. E. (1993). Cadmium uptake in gills of the mollusc *Crassostrea virginica* and inhibition by Ca channel blockers. *Aquatic Toxicology, 24,* 195–206.

Ruddell, C. L. (1971). Elucidation of the nature and function of the granular oyster amebocytes through histochemical studies of normal and traumatized oyster tissues. *Histochemie,* 26, 98–112.

SARF (Scottish Aquaculture Research Forum). (2010). *Review and cost-benefit analysis for industry of reduced depuration times for the mussel Mytilus edulis.* UK: Scottish Aquaculture Research Forum. p. 97.

Simkiss, K. (1981). Cellular discrimination processes in metal accumulating cells. *Journal of Experimental Biology, 94,* 317–327.

Suter, G. (2007). Freshwater bivalve ecotoxicology. In J. L. Farris, & J. H. Van Hassel (Eds.), *Integrated Environmental Assessment and Management,* 3(4), 568–569.

Suzuki, K. T., & Suzuki, T. (1996). An introduction to clinical aspects of toxicity. In W. Chang (Ed.), *Toxicology of metals* (pp. 333–335). Boca Raton: LCRC Lewis Publishers.

Thorsen, W. A., Cope, W. G., & Shea, D. (2007). Toxicokinetics of environmental contaminants in freshwater bivalves. In J. L. Farris, & J.H. Van Hassel (Eds.), *Freshwater bivalves ecotoxicology.* Boca Raton, FL: CRC Press. p. 169–214.

USEPA (US Environmental Protection Agency). (2007). *Method 7473 – Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrophotometry.* Washington, DC, USA: United States Environmental Protection Agency. p. 17.

Uzaki, N., Kai, M., Aoyama, H., & Suzuki, T. (2003). Changes in mortality rate and glycogen content of the Manila clam *Ruditapes philippinarum* during the development of oxygen-deficient waters. *Fisheries Science, 69,* 936–943.

Venkataraman, R., & Chari, S. D. T. (1951). Studies on oysters and clams – biochemical variations. *Indian Journal of Medical Research, 39,* 533–541.

Viles, P., & Silverman, J. (1949). Determination of starch and cellulose with anthrone. *Journal of Analytical Chemistry, 21,* 950–953.

Wang, Y., & Evans, R. D. (1993). Influence of calcium concentrations on cadmium uptake by the freshwater mussel *Ellptio complanata*. *Canadian Journal of Fisheries and Aquatic Sciences, 50(12),* 2591–2596.

Waykar, B., & Deshmukh, G. (2012). Evaluation of bivalves as bioindicators of metal pollution in freshwater. *Bulletin of Environmental Contamination and Toxicology, 88,* 48–53.

Williams, R. J. P. (1981). Physico-chemical aspects of inorganic element transfer through membranes. *Philosophical Transactions of the Royal Society B: Biological Sciences, 294,* 57–74.

Zuykov, M., Pelletier, E., & Harper, D. A. T. (2013). Bivalve mollusks in metal pollution studies: from bioaccumulation to biomonitoring. *Chemosphere, 93,* 201–208.

Supplementary data

Table S1. Results of two-way ANOVA evaluating the effects of depuration time and species (RP, MG and SP) on glycogen content, macro (S, Cl, K and Ca), trace (Fe, Zn, Br, Cu, Se, Rb and Sr) and toxic elements (Hg, Cd, Pb and As) concentration.

Parameters	df	MS	F	p	Parameters	df	MS	F	p
Glycogen					**Cu**				
Depuration time (DT)	4	1.3	41.0	< 0.001*	*Depuration time (DT)*	4	0.186	6.2	0.006*
Species (S)	2	10.6	327.1	< 0.001*	*Species (S)*	2	6.3	212.7	< 0.001*
DT x S	8	0.385	11.8	< 0.001*	*DT x S*	8	0.140	4.7	0.008*
Error	15	0.032			*Error*	12	0.030		
S					**Se**				
Depuration time (DT)	4	203865	2.9	0.049*	*Depuration time (DT)*	4	0.070	2.2	0.121
Species (S)	2	6929982	99.6	< 0.001*	*Species (S)*	2	3.5	108.0	< 0.001*
DT x S	8	65376	0.939	0.514	*DT x S*	8	0.081	2.5	0.058
Error	15	69605			*Error*	15	0.032		
Cl					**Rb**				
Depuration time (DT)	4	7885550	16.9	< 0.001*	*Depuration time (DT)*	4	1.6	32.4	< 0.001*
Species (S)	2	53034073	113.5	< 0.001*	*Species (S)*	2	1.5	29.8	< 0.001*
DT x S	8	1538769	3.3	0.022*	*DT x S*	8	0.346	6.8	0.001*
Error	15	467375			*Error*	15	0.050		
K					**Sr**				
Depuration time (DT)	4	12464	0.475	0.753	*Depuration time (DT)*	4	8.4	9.9	0.002*
Species (S)	2	513719	19.6	< 0.001*	*Species (S)*	2	81.9	97.6	< 0.001*
DT x S	8	23745	0.906	0.536	*DT x S*	8	7.7	9.1	0.001*
Error	15	26215			*Error*	10	0.840		
Ca					**Hg**				
Depuration time (DT)	4	29153	2.2	0.120	*Depuration time (DT)*	4	0.000	9.5	< 0.001*
Species (S)	2	154350	11.6	< 0.001*	*Species (S)*	2	0.003	197.2	< 0.001*
DT x S	8	20849	1.6	0.217	*DT x S*	8	0.000	16.6	< 0.001*
Error	15	13332			*Error*	15	0.000		
Fe					**Cd**				
Depuration time (DT)	4	44286	71.1	< 0.001*	*Depuration time (DT)*	4	0.001	7.4	0.002*
Species (S)	2	322396	517.9	< 0.001*	*Species (S)*	2	0.040	250.2	< 0.001*
DT x S	8	14927	24.0	< 0.001*	*DT x S*	8	0.001	7.6	< 0.001*
Error	15	622.5			*Error*	15	0.000		
Zn					**Pb**				
Depuration time (DT)	4	934.3	14.4	< 0.001*	*Depuration time (DT)*	4	0.148	192.4	< 0.001*
Species (S)	2	50530	777.053	< 0.001*	*Species (S)*	2	2.7	3467.8	< 0.001*
DT x S	8	579.6	8.9	< 0.001*	*DT x S*	8	0.080	103.6	< 0.001*
Error	15	65.0			*Error*	15	0.001		
Br					**As**				
Depuration time (DT)	4	583.6	77.9	< 0.001*	*Depuration time (DT)*	4	0.568	2.7	0.048*
Species (S)	2	11156	1488.7	< 0.001*	*Species (S)*	2	22.0	106.4	< 0.001*
DT x S	8	493.4	65.8	< 0.001*	*DT x S*	8	0.365	1.8	0.164
Error	15	7.5			*Error*	15	0.207		

Asterisk indicates significant differences ($p < 0.05$).

Table S2. Results from Pearson correlation coefficients between macro, trace and toxic elements of RP, MG and SP.

	RP		MG		SP	
Correlations tested	***r***	***p*-value**	***r***	***p*-value**	***r***	***p*-value**
S – Fe	-0.292	0.414	-0.677	**0.031***	0.406	0.278
S – Zn	0.682	**0.030***	0.414	0.234	0.626	0.072
S – Br	-0.501	0.140	-0.793	**0.006***	-0.128	0.743
S – As	-0.585	0.075	0.134	0.713	0.768	**0.016***
Cl – Fe	-0.476	0.164	0.141	0.698	-0.667	**0.050***
Cl – Br	0.304	0.394	0.527	0.117	0.838	**0.005***
Cl – Rb	-0.530	0.115	-0.398	0.254	-0.821	**0.007***
Cl – Pb	-0.589	0.073	-0.745	**0.013***	-0.494	0.177
K – Rb	-0.721	**0.019***	-0.859	**0.001***	0.188	0.628
K – Sr	0.150	0.679	-0.464	0.176	-0.700	**0.036***
K – Pb	-0.699	**0.024***	-0.448	0.194	0.258	0.502
K – As	-0.643	**0.045***	-0.081	0.823	0.372	0.325
Ca – Br	-0.787	**0.007***	0.397	0.256	0.350	0.356
Ca – Sr	0.348	0.324	0.390	0.265	0.718	**0.029***
Ca – Hg	-0.777	**0.008***	-0.057	0.877	0.508	0.163
Ca – Cd	-0.636	**0.048***	-0.348	0.325	0.535	0.138
Ca – As	-0.666	**0.036***	0.759	**0.011***	0.264	0.492
Fe – Br	0.432	0.212	0.883	**0.001***	-0.862	**0.003***
Fe – Rb	0.857	**0.002***	0.201	0.578	0.860	**0.003***
Fe – Cd	-0.210	0.561	-0.767	**0.010***	0.458	0.215
Fe – Pb	0.856	**0.002***	0.300	0.399	0.786	**0.012***
Zn – Cd	-0.561	0.092	0.702	**0.024***	-0.402	0.284
Zn – As	-0.273	0.446	0.081	0.824	0.747	**0.021***
Br – Cu	0.822	**0.004***	0.132	0.716	-0.130	0.738
Br – Se	0.708	**0.022***	-0.278	0.436	-0.013	0.973
Br – Rb	0.558	0.094	0.170	0.638	-0.806	**0.009***
Br – Sr	-0.012	0.974	0.580	0.079	0.714	**0.031***
Br – Hg	0.867	**0.001***	-0.382	0.275	-0.011	0.977
Br – Cd	0.520	0.123	-0.826	**0.003***	-0.210	0.588
Br – As	0.847	**0.002***	0.233	0.518	-0.172	0.657
Cu – Se	0.766	**0.010***	0.119	0.744	-0.330	0.385
Cu – Hg	0.705	**0.023***	0.152	0.675	0.540	0.134
Cu – As	0.734	**0.016***	0.432	0.212	0.478	0.193
Se – Hg	0.856	**0.002***	-0.101	0.781	-0.086	0.827
Se – As	0.809	**0.005***	0.547	0.102	0.067	0.864
Rb – Pb	0.939	**0.000***	0.658	**0.039***	0.826	**0.006***
Hg – As	0.771	**0.009***	0.245	0.495	0.804	**0.009***
Cd – Pb	0.049	0.893	0.219	0.544	0.790	**0.011***
CI – S	0.685	**0.029***	0.583	0.077	0.262	0.496
CI – Zn	0.467	0.173	0.653	**0.041***	0.171	0.660
CI – Cd	-0.344	0.330	0.796	**0.006***	0.236	0.541
Glycogen – S	-0.318	0.371	-0.729	**0.017***	-0.057	0.884
Glycogen – Ca	-0.761	**0.011***	0.287	0.421	-0.455	0.218
Glycogen – Fe	-0.067	0.853	0.830	**0.003***	0.609	0.082
Glycogen – Br	0.639	**0.047***	0.945	**<0.001***	-0.533	0.139
Glycogen – Hg	0.673	**0.033***	-0.176	0.626	-0.436	0.241
Glycogen – Cd	0.860	**0.001***	-0.859	**0.001***	0.084	0.830

CHAPTER 7

Ecophysiology of native and alien invasive clams in an ocean warming context

Anacleto P., Maulvault A.L., Lopes V.M., Repolho T., Diniz M., Nunes M.L., Marques A. and Rosa R.

(2014)

Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology, 175: 28–37
DOI: 10.1016/j.cbpa.2014.05.003

Comparative Biochemistry and Physiology, Part A 175 (2014) 28–37

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Ecophysiology of native and alien-invasive clams in an ocean warming context

Patrícia Anacleto [a,b,c,*], Ana Luísa Maulvault [b,c], Vanessa M. Lopes [a], Tiago Repolho [a], Mário Diniz [d], Maria Leonor Nunes [b,c], António Marques [b,c], Rui Rosa [a]

[a] Laboratório Marítimo da Guia, Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Av. Nossa Senhora do Cabo 939, Cascais 2750-374, Portugal
[b] Divisão de Aquacultura e Valorização (DivAV), Instituto Português do Mar e da Atmosfera (IPMA, I.P.), Av. Brasília, Lisboa 1449-006, Portugal
[c] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Rua dos Bragas, 289, Porto 4050-123, Portugal
[d] REQUIMTE, Departamento de Química, Centro de Química Fina e Biotecnologia, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica 2829-516, Portugal

ARTICLE INFO

Article history:
Received 16 January 2014
Received in revised form 4 May 2014
Accepted 7 May 2014
Available online 15 May 2014

Keywords:
Biological invasions
Metabolism
Oxidative stress
Ruditapes decussatus
Ruditapes philippinarum
Thermal tolerance

ABSTRACT

Both climate change and biological invasions are among the most serious global environmental threats. Yet mechanisms underlying these eventual interactions remain unclear. The aim of this study was to undertake a comprehensive examination of the physiological and biochemical responses of native (*Ruditapes decussatus*) and alien-invasive (*Ruditapes philippinarum*) clams to environmental warming. We evaluated thermal tolerance limits (CTMax), routine metabolic rates (RMRs) and respective thermal sensitivity (Q_{10} values), critical oxygen partial pressure (P_{crit}), heat shock response (HSP70/HSC70 levels), lipid peroxidation (MDA build-up) and antioxidant enzyme [glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD)] activities. Contrary to most studies that show that invasive species have a higher thermal tolerance than native congeners, here we revealed that the alien-invasive and native species had similar CTMax values. However, warming had a stronger effect on metabolism and oxidative status of the native *R. decussatus*, as indicated by the higher RMRs and HSP70/HSC70 and MDA levels, as well as GST, CAT and SOD activities. Moreover, we argue that the alien-invasive clams, instead of up-regulating energetically expensive cellular responses, have evolved a less demanding strategy to cope with short-term environmental (oxidative) stress-pervasive valve closure. Although efficient during stressful short-term periods to ensure isolation and guarantee longer survival, such adaptive behavioural strategy entails metabolic arrest (and the enhancement of anaerobic pathways), which to some extent will not be advantageous under the chronically warming conditions predicted in the future.

1. Introduction

Estuaries are among the most socio-economically and ecologically important coastal ecosystems and are known to be constantly subjected to anthropogenically derived pollution and natural stressors (e.g. temperature, pH, dissolved oxygen, salinity). Additionally, it has been recently shown that these coastal areas are warming at a faster rate in comparison to many other ecosystems (MacKenzie and Schiedek, 2007). According to the most recent report of the Intergovernmental Panel on Climate Change (IPCC), it is expected that by the end of the 21st century global mean surface temperature will increase by 0.3–4.8 °C (IPCC, 2013). Since many coastal organisms already live close to their thermal tolerance limits (Stillman and Somero, 2000; Helmuth et al., 2006; Hoegh-Guldberg et al., 2007; Tewksbury et al., 2008), ocean warming is expected to negatively impact their performance and survival (Rosa et al., 2012, 2013, 2014). As a consequence, this future thermal challenge will especially affect marine ectothermic organisms (e.g. bivalve molluscs) since their metabolism is constrained by oxygen supply at high (and low) temperatures with a progressive transition to an anaerobic mode of energy production [the "oxygen- and capacity-limitation of thermal tolerance" concept (Pörtner and Knust, 2007)]. The changes in aerobic scope of ectotherms with global warming are assumed to be not caused by lower levels of ambient oxygen, but rather by limited capacity of oxygen supply mechanisms (ventilatory and circulatory systems) to meet an animal's temperature-dependent oxygen demand (Pörtner and Knust, 2007).

There is increasing evidence that climate change will influence the dynamics of biological invasions, by affecting alien species entry pathways, establishment, spreading and colonization of new habitats (Capdevila-Argüelles and Zilletti, 2008). It is expected that, with global warming, inter-specific competition will occur with the more warm-adapted species replacing native species. The latter usually display lower thermal tolerance and, consequently, are unable to physiologically respond to extreme conditions (Calosi et al., 2008; Somero, 2010). Thus, the differential biological responses to future warming will have serious ecological (e.g. impact on ecosystem structure and function)

* Corresponding author at: Portuguese Institute of Sea and Atmosphere (IPMA, I.P.), Av. Brasília, 1449-006 Lisbon, Portugal. Tel.: +351 213 027 025; fax: +351 21 3015948.
E-mail address: panacleto@ipma.pt (P. Anacleto).

http://dx.doi.org/10.1016/j.cbpa.2014.05.003
1095-6433/

and economic implications (Hellmann et al., 2008). In Portugal, an example of native and alien species interactions is the closely related clam species: the grooved carpet shell clam *Ruditapes decussatus* (native species in the Atlantic and Mediterranean waters) and the Manila clam *Ruditapes philippinarum* (native species from the Indo-Pacific region). The latter was introduced in the beginning of the 1970s for aquaculture purposes in North European Atlantic and Mediterranean coastal waters (Flassch and Leborgne, 1992). This species has been recognized as one of the most successful invaders, being among the "100 worst invasive species in the Mediterranean" (Streftaris and Zenetos, 2006). Its high potential for dispersal, fast growth and ability to adapt new environments can have a major impact on local macrobenthic fauna and flora since it competes for food and space with other filter-feeding invertebrates (Otero et al., 2013).

Studies suggesting that invasive species are more eurythermal (ability to maintain physiological function over a wide range of temperatures) than native species have typically relied on latitudinal range as a proxy for both habitat temperature ranges and physiological temperature tolerance (Rejmánek, 1995, 1996; Rejmánek and Richardson, 1996). Yet although there is a growing interest in the study of physiological responses to environmental stress between alien-invasive and native organisms (Braby and Somero, 2006; Henkel et al., 2009; Lockwood and Somero, 2011; Zerebecki and Sorte, 2011; Coccia et al., 2013), the mechanisms underlying the interaction between climate change and successful biological invasions remain unclear.

The aim of this study was to undertake, for the first time, a comprehensive examination of the physiological and biochemical responses of native (*R. decussatus*) and alien-invasive (*R. philippinarum*) clams to thermal stress. More specifically, we investigated possible differences in: i) thermal tolerance limits (CTMax), ii) routine metabolic rates (RMRs), ii) thermal sensitivity (Q_{10} values), iii) critical oxygen partial pressure (P_{crit}), iv) heat shock response (HSP70/HSC70 levels), v) lipid peroxidation (MDA buildup) and vi) antioxidant enzyme [glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD)] activities.

2. Materials and methods

2.1. Specimen collection and stocking conditions

Specimens of *R. decussatus* (mean ± standard deviation; total weight: 6.7 ± 1.1 g; soft-tissue weight: 3.0 ± 0.9 g; shell length: 31.2 ± 1.8 mm; shell width: 14.8 ± 0.9 mm; shell height: 22.5 ± 1.2 mm) and *R. philippinarum* (mean ± standard deviation; total weight: 13.2 ± 3.9 g; soft-tissue weight: 2.3 ± 0.4 g; shell length: 35.3 ± 3.0 mm; shell width: 18.8 ± 1.8 mm; and shell height: 26.6 ± 2.3 mm) were harvested up to 10 and 30 m, respectively, through diving in active bivalve fishing areas of the Sado and Tagus estuaries (Western coast of Portugal; see Supplemental Fig. S1), during summer season (August–September 2012). Additionally, collection of sediment from clam harvest sites was also performed. After collection, *Ruditapes* sp. specimens were immediately transported in thermal boxes, to Guia Marine Laboratory (Centre of Oceanography, Faculty of Sciences, University of Lisbon, Cascais, Portugal) and randomly placed in 14 flat-bottom cylindrical fibreglass tanks (10 L capacity each and 4 cm bottom filled (height) with collection site sediment), within a recirculating aquaculture system, filled with natural seawater (0.2 μm and UV filtered). The recirculating aquaculture system was equipped with biological (ouriço®, Fernando Ribeiro Lda, Portugal), mechanical (100 μm, TMC-Iberia, Portugal) and physical (ReefSkimPro 850, TMC-Iberia, Portugal) filtration, in addition to UV disinfection (Vecton 600, TM-Iberia, Portugal). Ammonium and nitrite levels were determined daily by means of colorimetric test kits (Aquamerk, Merck Millipore, Germany) and kept below detectable levels. Additionally, salinity was daily checked and kept at 35 ± 1 PSU (V2 refractometer, TMC, UK). Water temperature was maintained at 22.0 ± 0.2 °C, by means of a water chiller (Frimar, Fernando Ribeiro Lda, Portugal) and submersible heaters (300 W, Eheim GmbH & Co. KG, Germany), while pH was kept at 8.2 ± 0.1. Up and down pH regulation was performed through a CO_2 and filtered atmospheric air (soda lime) injection system, controlled by a Profilux control system (Profilux 3.1N, GHL, Germany). Photoperiod was kept to 14-h light–10-h dark. The acclimation temperature (22 °C) was chosen since it reflects the average thermal value that both clam species face throughout the summer months in the estuaries. Clams were acclimated during seven days and fed 4× a day, with a commercially available microalgal mix (*Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira* and *Nannochloropsis* spp.; Acuinuga, Coruña, Spain) with the exception for the day prior to the experimental assays (respirometry and thermal tolerance experiments).

2.2. Thermal tolerance limits

Thermal tolerance was determined by the dynamic method described in Mora and Ospína (2001). The measured parameter was the Critical Thermal Maximum (CTMax given in degrees Celsius), defined as the "arithmetic mean of the collective thermal points at which the end-point is reached" (Mora and Ospína, 2001).

In order to determine the CTMax, organisms were subjected to a thermostable bath and placed into separated plastic containers, 20 specimens of each species, comprising three replicates (total $n = 60$). The bath temperature was set to the acclimation temperature and maintained for 30 min. Thereafter, temperature was increased at a rate of 1 °C per 30 min and clams were observed continuously, until they reached the end-point. Every 30 min, seawater was aerated and temperature in each container was checked, using a digital thermometer (TFX 430, Ebro, Germany). Afterwards, for each temperature gradient (from 22 °C to the temperature at which 50% of the clams died—LT50) and species, four individuals were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent biochemical analyses. In order to distinguish between live and dead specimens, inactive individuals were mechanically stimulated. All dead clams showed the valves completely open and no reaction to the stimulus (end-point). Since environmental variables that could influence results (e.g. oxygen levels, salinity, pH, feeding and temperature) were monitored during the acclimation and experiments, it is assumed that the observed results were due to temperature.

2.3. Routine metabolic rates, valve closure behaviour and thermal sensitivity

Oxygen consumption rates (routine metabolic rates; RMRs) were determined according to previously established methods (Rosa and Seibel, 2008, 2010; Aurélio et al., 2013). Individual clams were placed within an intermittent flow-through respirometry system (250 mL chambers; Loligo Systems, Tjele, Denmark). Five specimens of each species were used per temperature (from 22 °C to LT50). Respiration chambers were placed in thermostable water baths (Lauda, Lauda-Königshofen, Germany) in order to control the temperature. Oxygen concentrations were recorded with Clarke-type O_2 electrodes (SI130 microcathode oxygen electrode, Strathkelvin instruments Limited, North Lanarkshire, Scotland) connected to a multichannel oxygen interface (929, Strathkelvin Instruments Limited, North Lanarkshire, Scotland), during 2 h for each temperature gradient. System calibration was performed using oxygen-saturated seawater and checked for electrode drift before each run and at each experimental temperature. Due to weight dissimilarity between specimens of both species, RMRs were standardized to 2.5 g of soft-tissue wet weight assuming a scaling coefficient of −0.25 (3/4 power law; see also Rosa et al., 2009).

During respirometry runs, valve closure behaviour was also monitored, i.e., clams were continuously observed in order to detect opened

and closed valves. For each species, thermal sensitivity (Q_{10}) was determined using the standard equation:

$$Q10 = \left[\frac{R(T_2)}{R(T_1)}\right]^{\frac{10}{T_2-T_1}} \quad (1)$$

where $R(T_2)$ and $R(T_1)$ represent the oxygen consumption rates at temperatures T_2 and T_1, respectively. Q_{10} values were calculated for temperature intervals ranging from 22–24 to 40–LT50 °C (every 2 °C).

Additionally and for specific temperatures (low — 22 °C, intermediate — 30 °C and high temperature — corresponding to LT50), trials were prolonged up to 24 h to quantify the critical oxygen partial pressures (P_{crit}; as described in Rosa and Seibel, 2010; Rosa et al., 2013).

2.4. Biochemical analyses

2.4.1. Preparation of tissue extracts for heat shock proteins, lipid peroxidation and antioxidant enzymes

Quadruplicate homogenates were prepared using frozen soft-tissue of each clam. All samples were homogenized in 3 mL of homogenization buffer (phosphate-buffered saline solution, PBS, pH 7.3: 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4) by using a Potter Elvehjem glass/PTFE tissue grinder (Wheaton Science Products, Millville, NJ). This procedure was carried out in an icebox, in order to keep the proteins stable and to prevent shifts in enzyme activity. Afterwards, all homogenates were centrifuged (15 min at 10,000 *g* at 4 °C) in 1.5 mL microtubes. Subsequently, supernatant fraction was collected into new microtubes and immediately frozen (−80 °C) until further biochemical analyses (heat shock proteins: HSP70/HSC70 expression; lipid peroxidation: MDA concentration; and antioxidant enzyme activities: SOD, CAT and GST). All analyses were carried out in triplicate.

In order to normalize the results, the Bradford assay (Bradford, 1976) was used to quantify the total protein in each sample. The analysis was carried out in 96-well microplates (Nunc-Roskilde, Denmark) by adding 190 μL of Bradford reagent in each well and 10 μL of each sample or standards. Afterwards, absorbance was read at 595 nm in a microplate reader (Bio Rad, Benchmark, USA). A calibration curve was created using bovine serum albumin (BSA; NZYTech, Lisbon, Portugal) standards.

2.4.2. Heat shock protein (HSP70/HSC70)

2.4.2.1. ELISA (enzyme-linked immunosorbent assay). The heat shock response (HSR) was assessed from heat shock protein (HSP70/HSC70) production using an enzyme-linked immunosorbent assay (ELISA) based on a protocol from Njemini et al. (2005). Briefly, homogenate supernatant was diluted in PBS (1:50), of which 50 μL was added to each well of a 96-well microplate. After overnight incubation (4 °C), microplates were 3× washed in 0.05% PBS–Tween-20 (Sigma-Aldrich, USA) and 200 μL of blocking solution (1% BSA, Sigma-Aldrich, USA) was added to each well. After incubation (37 °C, 90 min) and a microplate washing step (PBS–Tween-20, see above), the primary antibody (anti-HSP70/HSC70, Acris, USA), which detects 72 and 73 kDa proteins (molecular mass of inducible and constitutive isoforms, respectively), was added to each well (50 μL of 0.5 μg mL^{-1} solution per well) and then incubated at 37 °C for 90 min. After removal of the non-linked primary antibody (washing step with PBS–Tween-20), the secondary antibody (anti-mouse IgG, Fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was added to each well (50 μL of 1 μg mL^{-1} solution per well) and further incubated at 37 °C for 90 min. After another washing procedure (3×), 100 μL of substrate (SIGMA FAST™ *p*-nitrophenyl phosphate tablets, Sigma-Aldrich, USA) was added to each well and incubated at room temperature for 90 min. The reaction was stopped by adding 50 μL of NaOH solution (3 M) to each well and the absorbance was read at 405 nm in a 96-well microplate reader (Bio-Rad, Benchmark, USA). Heat shock protein quantification (HSP70/HSC70) in samples was calculated from a standard curve based on serial dilutions (0–1000 ng mL^{-1}) of purified HSP70 active protein (Acris, USA). Results were expressed in relation to total protein in the sample wet mass (μg HSP70/HSC70 μg^{-1} total protein).

2.4.2.2. Western blot. The confirmation of antibody specificity for tested species was carried out by western blot (WB) analysis by following the procedure described by Bolt and Mahoney (1997). Aliquots from samples containing 20 μg of total protein were added to sample buffer at a proportion of 1:1 (100 mM Tris at pH 6.8; 20% glycerol; 4% SDS; 0.002% bromophenol blue). The samples were then denatured in boiling water (3–5 min) and loaded into a 7.5% SDS–PAGE gel to resolve proteins. The gels were run in a Mini-PROTEAN® Cell (Bio-Rad, USA) at constant 120 V and 400 mA for 80 min. The gel was equilibrated in transfer buffer (25 mM Tris; 192 mM glycine; 10% methanol) for 15 min and the PVDF Immobilon-P membrane (Merck Millipore, USA) was wet in ultrapure water and soaked in transfer buffer. Next, the electro-blotting sandwich, containing sponges, filter paper, gel and PVDF membrane was assembled and electro-transferred (15 V, 90 mA, 16 h). After the transfer, the membrane was blocked in 5% BSA/PBS for 3 h in a shaker. Membranes were then washed in PBS 0.05% Tween-20 (PBST) for 10 min followed by one hour incubation (at 37 °C with constant agitation) with a primary monoclonal antibody (goat anti-mouse HSP70, Acris, USA) diluted 1/5000 in 1% milk/PBS. After washing with 0.05% PBST (5 min) the membrane was incubated for 2 h with an alkaline phosphatase conjugate secondary antibody (goat anti-mouse IgG-fab specific, Sigma-Aldrich, USA), diluted 1:10,000 in 1% BSA/PBS. The membrane was washed as above and developed with 1% (v/v) BCIP and 1% (v/v) NBT, until satisfactory results as per manufacturer instructions. The reaction was finished with 1 mM EDTA and images of the blots were obtained by scanning the developed membrane.

2.4.3. Lipid peroxidation (determination of malondialdehyde, MDA)

Lipid peroxidation assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978) and determined by quantifying a specific end-product of the oxidative degradation process of lipids, malondialdehyde (MDA), where thiobarbituric acid reacts with MDA yielding a fluorescent product that is detected spectrophotometrically at 532 nm. Briefly, 5 μL of each sample, already processed as described previously, was added to 45 μL of 50 mM monobasic sodium phosphate buffer. Then, 12.5 μL of sodium dodecyl sulfate (SDS 8.1%), 93.5 μL of trichloroacetic acid (20%, pH = 3.5) and 93.5 μL of thiobarbituric acid (1%) were added. To this mixture, 50.5 μL of Milli-Q grade ultrapure water (Milli-Q 185 Plus system, Millipore, Bedford, MA, USA) was added and the solution was mixed in a vortex for 30 s and incubated in boiling water for 10 min. Afterwards, microtubes were placed on ice to lower the temperature. Subsequently, 62.5 μL of Milli-Q water and 312.5 μL of n-butanol pyridine (15:1, v/v) were added and the solution was centrifuged at 7000 rpm for 5 min. Duplicates of 150 μL supernatant of each reaction were inserted into a 96-well microplate and absorbance was read (532 nm). To quantify lipid peroxidation, MDA concentrations were calculated based on an eight-point calibration curve (0–0.3 μM TBARS) using MDA bis-(dimethylacetal) standards (Merck, Germany). Results were expressed in relation to total protein in the sample wet weight (nmol min^{-1} μg^{-1} total protein).

2.4.4. Determination of antioxidant enzyme activities

2.4.4.1. Glutathione S-transferase (GST). GST activity was determined according to the procedure described by Habig et al. (1974) and optimised for a 96-well microplate (Rosa et al., 2012). Enzyme activity was determined spectrophotometrically by measuring the formation of the conjugate of glutathione (GSH) and l-chloro-2,4-dinitrobenzene (CDNB). The assay contained 200 mM L-glutathione (reduced), Dulbecco's PBS

and 100 mM CDNB solution. Equine liver GST (Sigma-Aldrich, Germany) was used as standard and positive control. In order to perform the assay, 180 μL of substrate solution was added to 20 μL of GST standard or sample in each well of a 96-well microplate (Nunc-Roskilde, Denmark). Absorbance (340 nm) was recorded, every minute during a 6 min time period, using a microplate reader. The increase in absorbance is directly proportional to the GST activity and the reaction rate was determined using the CDNB extinction coefficient of 0.0053 μM^{-1} cm^{-1}. Results were expressed in relation to total protein in the sample wet weight (nmol min^{-1} μg^{-1} total protein).

2.4.4.2. Catalase (CAT). Catalase (EC 1.11.1.6) activity was determined according to the procedures previously described (Aebi, 1983; Li and Schellhorn, 2007). Total reaction volume of 3 mL was composed of 50 mM potassium phosphate buffer (pH 7.0) and 12.1 mM H_2O_2 as a substrate, and the reaction was started by adding the sample. Peroxide consumption (extinction coefficient of 0.04 mM cm^{-1}) was monitored using a spectrophotometer (Helios, Unicam, UK), at 240 nm reading. Absorbance was measured each 15 s (120 s in total), at 25 °C. Standard catalase activity was measured using a bovine catalase solution (Sigma-Aldrich, Germany) of 1523.6 U mL^{-1} (positive control). Results were expressed in relation to total protein in the sample wet mass (nmol min^{-1} μg^{-1} total protein).

2.4.4.3. Superoxide dismutase (SOD). Superoxide dismutase (EC 1.15.1.1) activity was determined spectrophotometrically based on the method described by Sun et al. (1988) at 550 nm (25 °C). The assay contained 50 mM potassium phosphate buffer (pH 7.8), 3 mM ethylenediaminetetraacetic acid (EDTA), 3 mM xanthine solution, 0.75 mM nitroblue tetrazolium (NBT), 100 mU xanthine oxidase solution (XOD) and 1 U μL^{-1} SOD enzyme solution. Superoxide dismutase from bovine erythrocytes (Sigma-Aldrich, Germany) was used as standard and positive control. Results of the enzymatic assay are given in units of SOD activity per microgram of total protein in the sample wet weight (U μg^{-1} total protein), where one unit of SOD is defined as the amount of sample causing 50% inhibition of NBT reduction.

2.5. Statistical analyses

Data were analysed for normality and homogeneity of variances through Kolmogorov–Smirnov and Levene's tests, respectively. A one-way analysis of variance (ANOVA) or, when necessary, Kruskal–Wallis ANOVA was performed to detect significant differences. Subsequently, post-hoc tests (Tukey HSD and unequal N HSD) or a non-parametric multiple comparison test (Dunn's test) was performed. Additionally, two-way ANOVA was performed in order to detect statistically significant differences in routine metabolic rates (RMRs), heat shock protein expression (HSP70/HSC70), lipid peroxidation (MDA) and antioxidative enzyme (GST, CAT and SOD) activities between clam species and temperature. Statistical analyses were performed for a significance level of 0.05, using STATISTICA™ software (Version 7.0, StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results

3.1. Mortality, thermal tolerance limits and metabolic rate

Although mortality only occurred after 36 °C and 40 °C, in the native (*R. decussatus*) and alien-invasive (*R. philippinarum*) species, respectively (Fig. 1), LT50 values were similar between them (42 °C). Additionally, while the native species showed 100% of mortality at 44 °C and the alien-invasive at 46 °C, there were no significant differences between their CTMax values (Fig. 2; Mann–Whitney, $U = 1753$, $p > 0.05$).

Routine metabolic rates (RMRs) were significantly affected by temperature and species (two-way ANOVA, $F = 6.2$, $p < 0.001$ and $F = 141.4$, $p < 0.001$, respectively; Fig. 3; Table 1). Additionally, a

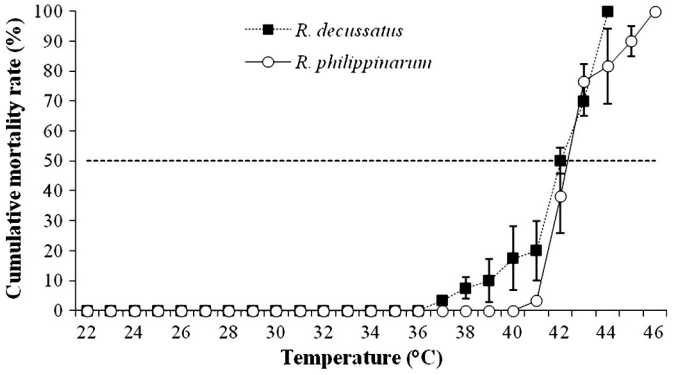


Fig. 1. Effect of environmental warming on the cumulative mortality rates (%) of native (*Ruditapes decussatus*) and alien-invasive (*Ruditapes philippinarum*) clams. The dashed line indicates the LT50 values.

temperature–species interaction was observed ($F = 5.6$, $p < 0.001$; Table 1). RMRs significantly increased with temperature, ranging from 5.33 ± 0.85 μmol O_2 g^{-1} h^{-1} (22 °C) to 9.42 ± 2.36 μmol O_2 g^{-1} h^{-1} (38 °C) for *R. decussatus*, and from 3.64 ± 0.61 μmol O_2 g^{-1} h^{-1} (22 °C) to 7.98 ± 1.13 μmol O_2 g^{-1} h^{-1} (30 °C) for *R. philippinarum*. Experimental species showed a distinct pattern regarding RMRs: the native species showed a significant decrease in RMRs after 39 °C (3.65 ± 1.01 μmol O_2 g^{-1} h^{-1}), while the alien-invasive clams revealed a significant decrease between 31 °C and 38 °C (3.05–1.21 μmol O_2 g^{-1} h^{-1}) followed by a noticeable augment towards the LT50 (2.49 ± 0.47 μmol O_2 g^{-1} h^{-1} at 42 °C; see horizontal dashed line in Fig. 1). Overall, *R. decussatus* always revealed higher RMR values in comparison to *R. philippinarum* at all temperatures (Fig. 3A, B).

The thermal sensitivity of RMRs, expressed as Q_{10} values, varied greatly across the temperature range and between both species (Fig. 4). Q_{10} values ranging between 1.5 and 3.0 were observed at the lower temperature intervals (22 °C–28 °C/30 °C) in both native and alien-invasive species. Then, Q_{10} values were kept below 1.5 reaching almost 0 (i.e. towards temperature independence of metabolism). Yet between 38 °C and 42 °C, the alien-invasive species showed surprisingly high Q_{10} values (between 4 and 9).

3.2. Valve closure behaviour and critical oxygen partial pressures

Under the environmental warming conditions tested, a quite different valve closure behaviour was observed between the studied species (Fig. 5). While the alien-invasive clam (*R. philippinarum*) generally remained completely closed when subjected to increasing water temperatures, the native species (*R. decussatus*) kept the valves opened

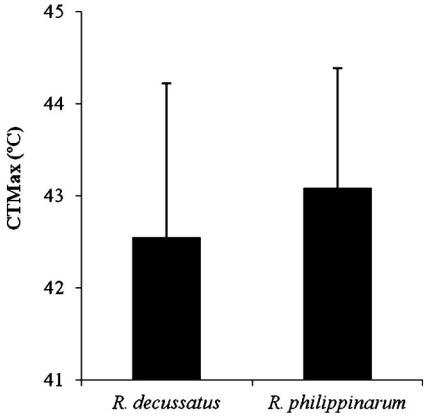


Fig. 2. Effect of environmental warming on the thermal tolerance limits (CTMax, °C) of native (*Ruditapes decussatus*, $n = 60$) and alien-invasive (*Ruditapes philippinarum*, $n = 60$) clams. Values represent mean ± SD.

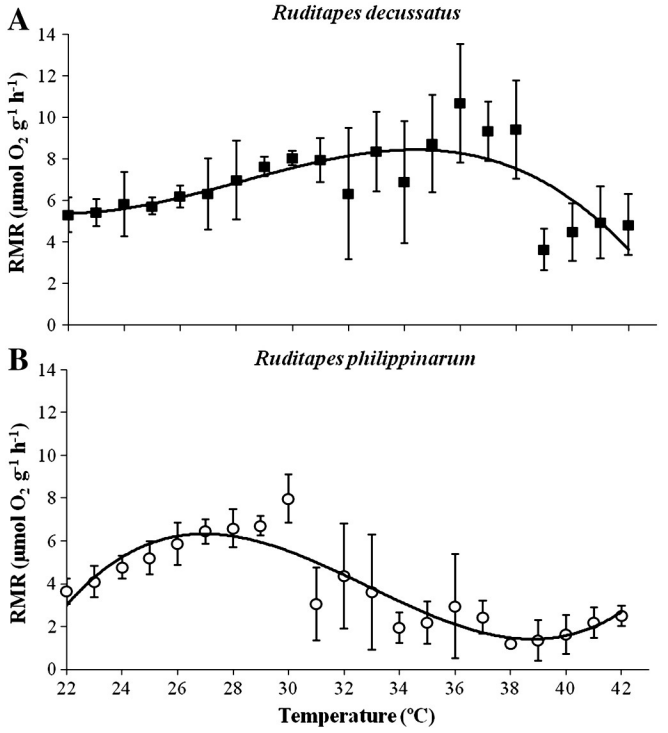


Fig. 3. Effect of environmental warming on the routine metabolic rates (RMRs, µmol O_2 g^{-1} h^{-1} wet weight) of: A) native (*Ruditapes decussatus*) and B) alien-invasive (*Ruditapes philippinarum*) clams. Values represent mean ± SD ($n = 5$ per each temperature). Rates were estimated (standardized) to 2.5 g animals assuming a scaling coefficient of −0.25. Black lines represent trendlines. See statistical details in Table 1.

throughout the entire temperature gradient (Fig. 5B), except between 32 and 35 °C where 20% of clams closed their valves (Fig. 5A). However, above 41 °C, all *R. philippinarum* specimens also opened the valves (Fig. 5B). Both clam species steadily reduced their oxygen uptake with decreasing PO_2 (Fig. 6), indicating an oxyconformer behaviour.

3.3. Heat shock response and lipid peroxidation

Heat shock response (HSP70/HSC70 levels) and lipid peroxidation (MDA levels) were significantly affected by temperature and between species (HSP70/HSC70: $F = 12.9$, $p < 0.001$ and $F = 1432.0$, $p < 0.001$, respectively; MDA: $F = 8.4$, $p < 0.001$ and $F = 57.2$, $p < 0.001$, respectively; Fig. 7A; Table 1). The specificity of HSP70 antibody in clams was confirmed trough western blot analysis, showing differences in HSP expression between the two clam species (Fig. 8). Heat shock protein levels significantly increased from 0.023 to 0.052 µg µg^{-1} total protein between 22 °C and 32 °C, but decreased steadily towards the LT50 temperature (42 °C) in native clams. Contrarily, HSP levels stayed approximately stable (≈0.015 µg µg^{-1} total protein) in the alien-invasive species. As previously observed for RMRs, *R. decussatus* always showed significantly higher HSP values in comparison to *R. philippinarum* (e.g. up to 6.8-fold higher at 28 °C) throughout the entire temperature gradient (more intense bands; Fig. 8).

Malondialdehyde (MDA) levels significantly changed with warming in both species, but with distinct patterns of variation (Fig. 7B). While *R. philippinarum* showed constant values up to 36 °C, increasing afterwards until reaching the LT50 temperature (≈0.002 nmol min^{-1} µg^{-1} total protein), *R. decussatus* revealed a peak of MDA buildup at 28 °C (>0.003 nmol min^{-1} µg^{-1} total protein), after which it decreased significantly attaining the lowest values at 38 °C and 42 °C (<0.001 nmol min^{-1} µg^{-1} total protein). Once more, *R. decussatus* showed significantly higher MDA values than *R. philippinarum*.

Table 1
Results of two-way ANOVA evaluating the effects of temperature and species (*Ruditapes decussatus* and *Ruditapes philippinarum*) on routine metabolic rates (RMRs), heat shock response (HSP70/HSC70 levels), lipid peroxidation (MDA levels) and glutathione S-transferase (GST), catalase (CAT) and superoxide-dismutase (SOD) activities.

Parameters	df	MS	*F*	*p*
RMRs				
Temperature (T)	20	15.7	6.2	<0.001*
Species (S)	1	357.1	141.4	<0.001*
T × S	20	14.1	5.6	<0.001*
Error	134	2.5		
HSP70/HSC70				
Temperature (T)	10	0.000	12.9	<0.001*
Species (S)	1	0.040	1432.0	<0.001*
T × S	10	0.000	14.7	<0.001*
Error	168	0.000		
MDA				
Temperature (T)	10	0.000	8.4	<0.001*
Species (S)	1	0.000	57.2	<0.001*
T × S	10	0.000	10.2	<0.001*
Error	75	0.000		
GST				
Temperature (T)	10	0.000	4.6	<0.001*
Species (S)	1	0.000	87.9	<0.001*
T × S	10	0.000	3.6	<0.001*
Error	135	0.000		
CAT				
Temperature (T)	10	0.002	33.8	<0.001*
Species (S)	1	0.073	1293.1	<0.001*
T × S	10	0.002	27.8	<0.001*
Error	169	0.000		
SOD				
Temperature (T)	10	0.000	93.3	<0.001*
Species (S)	1	0.004	1195.6	<0.001*
T × S	10	0.000	44.2	<0.001*
Error	179	0.000		

* Significant differences ($p < 0.05$).

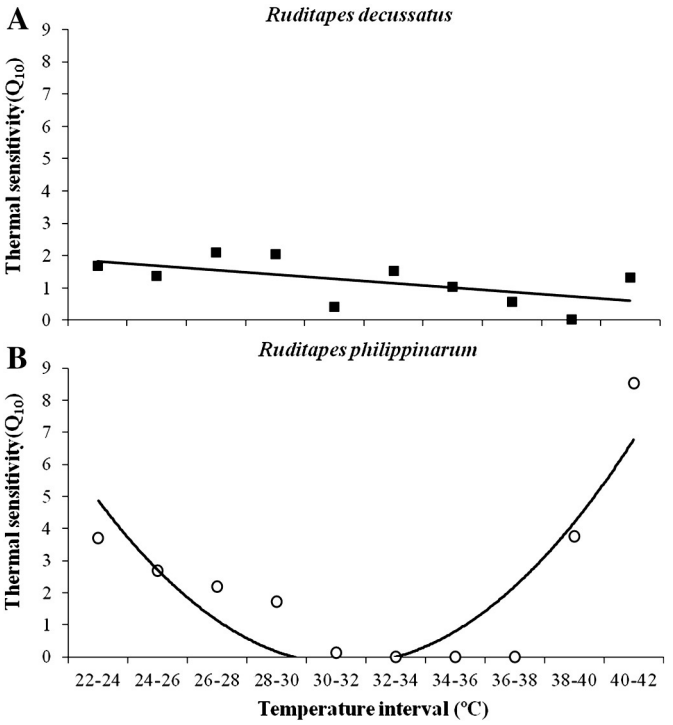


Fig. 4. Effect of environmental warming on thermal sensitivity (Q_{10} values) of: A) native (*Ruditapes decussatus*) and B) alien-invasive (*Ruditapes philippinarum*) clams. Black lines represent trendlines.

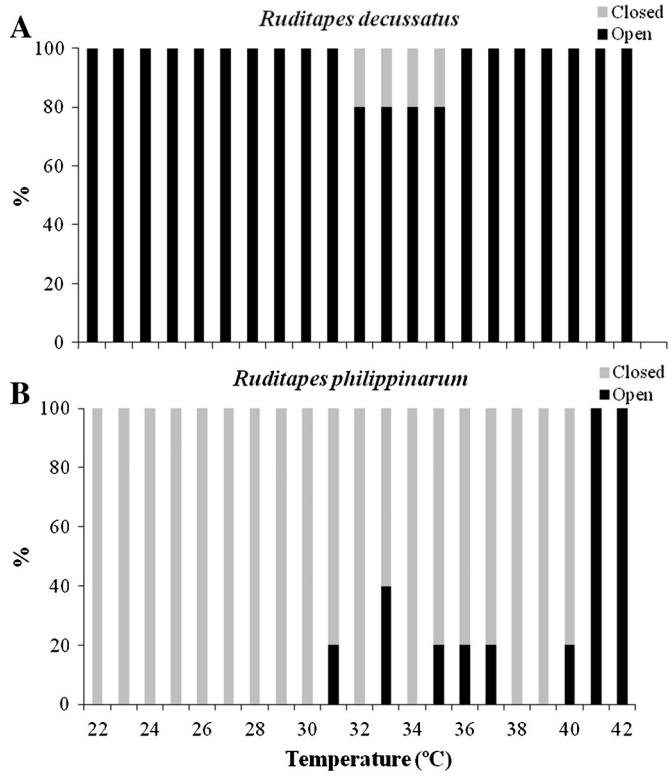


Fig. 5. Effect of environmental warming on the valve closure behaviour (as % of open and closed valves) of: A) native (*Ruditapes decussatus*) and B) alien-invasive (*Ruditapes philippinarum*) clams ($n = 5$ per each temperature).

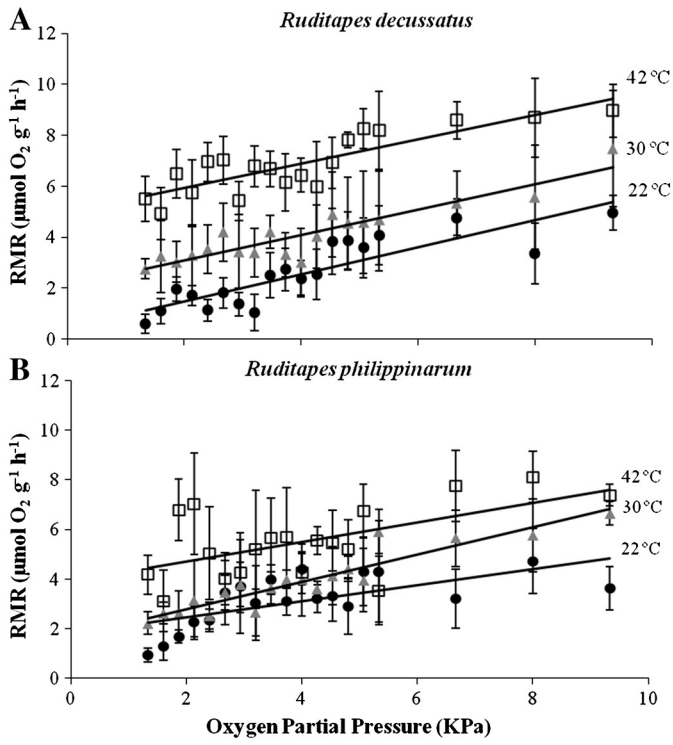


Fig. 6. Effect of environmental warming on the routine metabolic rates (RMRs, µmol O_2 g^{-1} h^{-1} wet weight) as a function of oxygen partial pressure (PO_2; KPa) in: A) native (*Ruditapes decussatus*) and B) alien-invasive (*Ruditapes philippinarum*) clams. Values represent mean ± SD ($n = 5$ per each temperature). Black lines represent trendlines. See statistical details in Table 1.

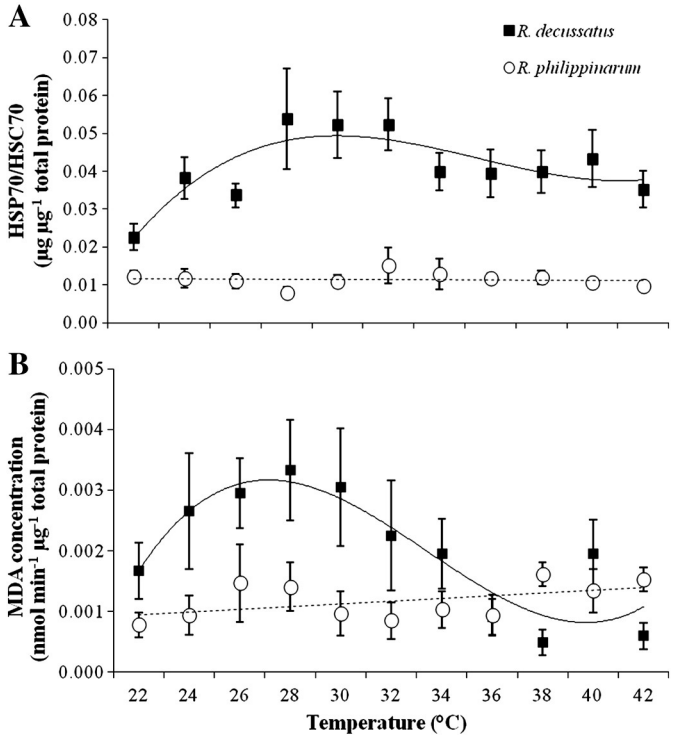


Fig. 7. Effect of environmental warming on: A) heat shock response (HSP70/HSC70, µg $µg^{-1}$ total protein) and B) lipid peroxidation (expressed as MDA concentration, nmol min^{-1} $µg^{-1}$ total protein) of native (*Ruditapes decussatus*) and alien-invasive (*Ruditapes philippinarum*) clams. Values represent mean ± SD ($n = 4 \times 3$ per each temperature). Black lines represent trendlines. See statistical details in Table 1.

3.4. Antioxidant enzyme activities

The antioxidant enzyme activities were also significantly affected by temperature and between species (Fig. 9; see Table 1). *R. decussatus* revealed higher GST, CAT and SOD activities in comparison to *R. philippinarum* in nearly all temperatures tested (Fig. 9). Moreover, GST, CAT and SOD activity levels were, to some extent, stable in the alien-invasive species ($p > 0.05$), while the native species displayed different patterns of variation (more or less stable in GST, modal in SOD and trimodal in CAT). However, evidence that antioxidant enzyme activities in native clams tended to be higher at temperatures below 30 °C was noticeable (GST: 0.011 nmol min^{-1} $µg^{-1}$ total protein, at 30 °C; CAT: 0.10 nmol min^{-1} $µg^{-1}$ total protein, at 24 °C; and SOD: 0.04 U $µg^{-1}$ total protein, at 30 °C).

4. Discussion

4.1. Mortality and thermal tolerance limits

Invasive species tend to inhabit locations with broader temperature ranges and higher maximum temperatures than native species, and therefore, large distributional range has been suggested as a general characteristic of invasion success (Rejmánek, 1996, 2000). Moreover,

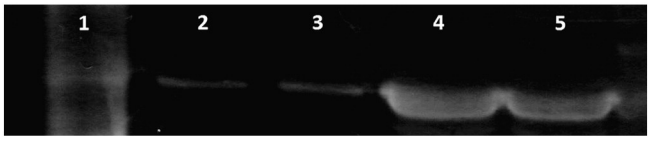


Fig. 8. Representative western blot from both clam species exposed to 30 °C, showing differences in HSP70 levels between these species. Legend: a) lane 1: purified HSP70 active protein; b) lanes 2 and 3: HSP70 from *Ruditapes philippinarum*; c) lanes 4 and 5: HSP70 from *Ruditapes decussatus*.

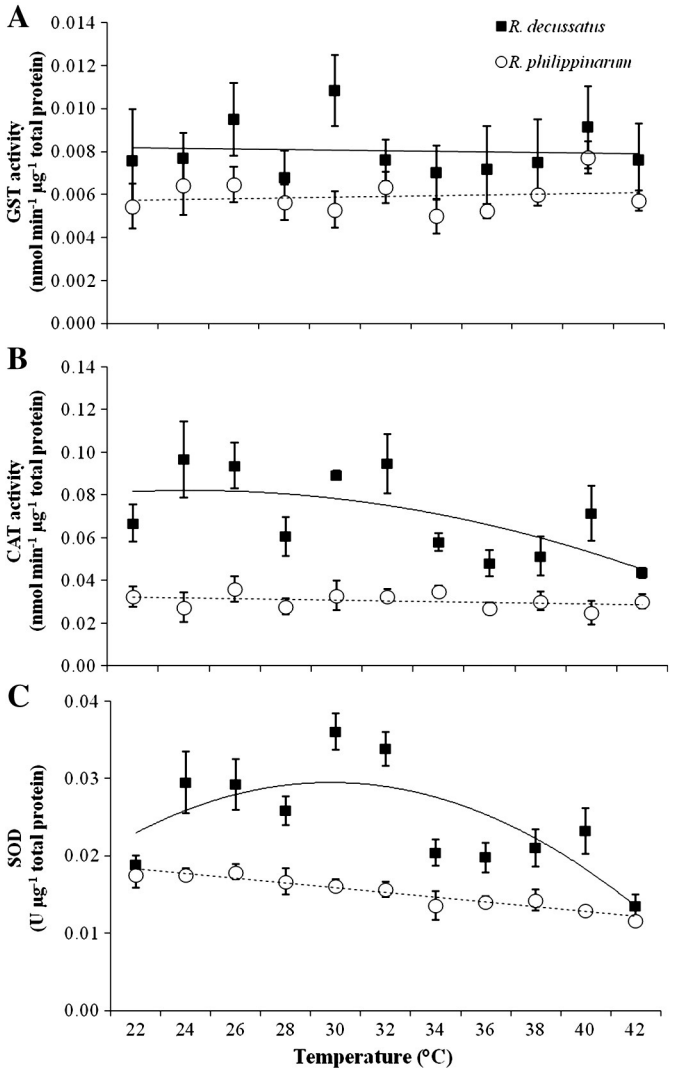


Fig. 9. Effect of environmental warming on the antioxidant enzyme activities of native (*Ruditapes decussatus*) and alien-invasive (*Ruditapes philippinarum*) clams. A) Glutathione-S-transferase (GST, nmol min^{-1} μg^{-1} total protein), B) catalase (CAT, nmol min^{-1} μg^{-1} total protein) and C) superoxide-dismutase (SOD, U μg^{-1} total protein). Values represent mean ± SD ($n = 4 \times 3$ per each temperature). Black lines represent trendlines. See statistical details in Table 1.

as invasive species have to pass through several abiotic (and biotic) filters in the multistage invasion process, organisms with broader physiological tolerances may be more likely to survive and become established as invaders (Mack et al., 2000; Olyarnik et al., 2009). Thus, global warming could be a boon for invasive species in marine ecosystems (Stachowicz et al., 2002). Nonetheless, while some argue that species with greater thermal tolerances will be better able to cope with global warming (Calosi et al., 2008), others claim that they will be at a disadvantage because most of them already live close to their absolute tolerance limits (Stillman and Somero, 2000; Compton et al., 2007; Somero, 2010; Sorte et al., 2011) and display lower acclimation potentials (Somero, 2010).

Lockwood and Somero (2011) also demonstrated that the invasive mussel *Mytilus galloprovincialis* had a higher thermal tolerance than its native congener *Mytilus trossulus*. Similarly, Sorte et al. (2010) revealed that invasive species of marine fouling community (namely bryozoans and tunicates) were more tolerant to higher temperatures (higher LT50) than native species, suggesting that climate change will have a disproportionately negative impact on native species. In contrast, in the present study, the alien-invasive clam *R. philippinarum* was not more tolerant to environmental warming than the native *R. decussatus*, as shown by the similar LT50 and CTMax values (Figs. 1 and 2, respectively). It would be expected that the former species would show greater physiological plasticity since it is found at shallower habitat depths (<10 m; i.e. subject to greater environmental variations) than the native one (up to depths of 20 m). Thus, we argue that life-history strategies (e.g. reproductive traits) may play an important role in the invasion success of *R. philippinarum*. Successful invaders can face the ecological pressure posed by their new environment by allocating reproductive efforts over several breeding events (Sol et al., 2012). In fact, *R. philippinarum* has a more extended breeding period and a greater number of spawning events than *R. decussatus* (Laruelle et al., 1994), which may confer on them an adaptive advantage.

4.2. Metabolic rate, valve closure behaviour and critical oxygen partial pressures

As expected, each species' metabolic rate increases with environmental warming, namely between 22–38 °C and 22–30 °C for native and alien-invasive clams, respectively (Fig. 3). The respective Q_{10} values indicated active metabolic regulation (Hochachka and Somero, 2002) and that clams were not under thermal stress within these intervals (Fig. 4). Interestingly, native species exhibits a peak of RMRs between 33 °C and 35 °C (around 9 µmol O_2 g^{-1} h^{-1}), while for the alien-invasive clams it was between 26 and 28 °C (roughly 6–7 O_2 g^{-1} h^{-1}). However, at elevated temperatures there was a significant decrease in RMRs for both clam species (Fig. 3A, B). It is worth noting that this break point was significantly different between species (38 °C and 30 °C for the native and alien-invasive species, respectively). The concomitant decline in Q_{10} values (to below 1.5) indicated active metabolic depression towards temperature-independent metabolism (Fig. 4). This is a well-known energy conserving strategy in marine molluscs (Sokolova and Pörtner, 2001), especially for those experiencing major variations in environmental temperature (Widdows, 1976). It is noteworthy that a drastic shift to extremely high Q_{10} values at 40–42 °C was found in the alien-invasive species (but not in the native one). Interestingly, this shift seemed to be related to sudden behaviour change—i.e., all alien-invasive clams opened their valves under such harsh thermal conditions (Fig. 5B). Indeed, a striking inter-specific difference between clam species was the valve closure behaviour. While the native species showed valve gaping at all temperatures, the alien-invasive clams tightly close their valves during almost the entire warming gradient. This behaviour restricts gas exchange, reduces aerobic metabolism (Anestis et al., 2010) and may be an important strategy for longer survival under harsh environmental conditions. In fact, previous studies have indicated that metabolic depression is usually accompanied by valve closure in bivalves (de Zwaan et al., 1980; Ortmann and Grieshaber, 2003; Anestis et al., 2007). Nevertheless, this is a passive strategy of thermal tolerance in most marine ectotherms and occurs at the expense of reduced aerobic scope for activity (Pörtner, 2002), with a progressive transition to an anaerobic mode of energy production (Pörtner et al., 2004; Pörtner and Knust, 2007).

Based on the relationship between RMRs and oxygen partial pressure (PO_2), both clam species may be considered oxyconformer organisms (Fig. 6). In order to cope with the impacts of abiotic factors (e.g. warming and hypoxia) on energy production, several marine organisms have developed an oxyconformer strategy, i.e., they regulate their O_2 consumption rate in a nearly linear function to the minimum O_2 levels encountered in their environments (Grieshaber et al., 1994; Childress and Seibel, 1998; Boutilier, 2001). In other words, their critical oxygen partial pressure (P_{crit}) values are generally lower than the minimum O_2 level they encounter to favour diffusion pathways and O_2 uptake. Other bivalves, such as the mussel *M. galloprovincialis*, are designated as oxyregulators (Jansen et al., 2009), since they are able to keep their oxygen consumption independent of the ambient oxygen tension down to a certain PO_2, below which regulation ceases (Hardewig et al., 1991). Contrary to mussels, clam species are capable of breathing

at the sediment surface as well as burrowing several centimetres deep in the sediment. In fact, while *R. decussatus* is known to be capable of burrowing in the sediment to a depth of 15–20 cm (FAO, 2013), *R. philippinarum* usually burrows 3–4 cm deep (Kondo, 1987; Jones et al., 1993). During periods of burrowing, which may last several days, the PO_2 can decline to almost 0 kPa, which causes a state of metabolic rate depression and leads to anaerobiosis (Taylor, 1976).

4.3. Heat shock response, lipid peroxidation and antioxidant enzyme activities

When temperature increases, clams are subjected to oxidative damage associated with ROS production (Bagnyukova et al., 2007; Lushchak, 2011) simply by the enhancement of mitochondrial respiration (Abele et al., 2002). Concomitantly, heat shock response (HSR) is regulated to promote thermoprotection in a gradually warming environment, by refolding of denatured proteins and preventing further protein unfolding and aggregation (Rosa et al., 2012; Lopes et al., 2013; Teixeira et al., 2013). Yet only native clams elicited a HSR (Fig. 7A) and significantly changed the activity of antioxidant enzymes (Fig. 8). Based on Tomanek (2008), we argue that the T_{on} (onset temperature), T_{peak} (temperature of maximal induction) and T_{off} (cessation temperature of HSR) for the native species were 24, 28 and 34 °C, respectively. Above T_{off}, extreme hyperthermia also seemed to reduce antioxidant enzymatic defences (Fig. 8; Kregel, 2002; Abele and Puntarulo, 2004); at that stage, non-enzymatic defences (e.g. vitamins E, C, and A, glutathione, ascorbate, carotenes and ubiquinol$_{10}$) may be particularly important (Filho, 1996) and may explain the lowering of MDA concentrations (Fig. 7B). MDA concentrations are indicative of the enhancement of ROS action in an organism's lipids (known as peroxidation), a process considered to be one of the most frequent cellular injury mechanisms (Lesser, 2006). Additionally, the decrease of CAT activity in native clams (Fig. 8B) indicates a reduced activity to protect the cells from the accumulation of H_2O_2, while the decrease in SOD activity (Fig. 8C) can be explained by the reduced production of this enzyme's substrate (O_2^-) as a consequence of lower oxygen availability at higher temperatures.

As in our native species, similar HSR were observed in gastropods (Tomanek, 2002, 2010) and crustaceans (Jost et al., 2012). According to Tomanek and Somero (1999), at any temperature above T_{peak}, HSP synthesis cannot match the increasing cellular thermal insult, suggesting that T_{off}'s relevance is limited under natural conditions. Moreover, HSR is a highly ATP-dependent mechanism and thus at warming conditions species may become energy limited. As we previously mentioned, further warming leads to a transition to an anaerobic mode of mitochondrial metabolism and progressive insufficient energy at the cellular level (Pörtner, 2002, 2010). In this sense, at LT50 (Fig. 1) there may not be sufficient cell ATP to mount the HSP70 response.

On the other hand, HSR, lipid peroxidation and antioxidant enzyme activities were quite stable throughout the entire temperature gradient in the alien-invasive clams (Figs. 7 and 8). Different HSR responses were found by Hofmann and Somero (1996) and Braby (2004), who demonstrated greater induction of HSP70 in the invasive mussel *M. galloprovincialis* than its native *M. trossulus*. Zerebecki and Sorte (2011) also found higher HSP70 levels in the invasive tunicate *Diplosoma listerianum* than in the native *Distaplia occidentalis*, and assumed it to be related with the relatively greater temperature tolerance of the invasive species. Yet the strategy commonly used by species may depend not only on their thermal history, but also on species-specific traits and spatial scale (Hofmann and Somero, 1996; Tomanek, 2008). Other studies also demonstrated different magnitudes of stress responses (both physiologically and at a molecular level) according to their sex (Øverli et al., 2006), age/size (Hall et al., 2000) and habitat (Timofeyev et al., 2009). In contrast to mussels, we argue that the alien-invasive clams, instead of up-regulating energetically expensive (heat shock and antioxidant) cellular responses as the native species does, have adopted a less energy-demanding (behavioural) strategy to cope with short-term environmental (oxidative) stress — burrowing with pervasive valve closure (Fig. 5B). Yet one should keep in mind that such adaptive strategy (valve closure) entails metabolic depression and the enhancement of anaerobic pathways, which will not be advantageous under the chronically warming conditions predicted in the future (Rosa et al., 2012, 2013, 2014).

5. Conclusions

Extreme high temperature (heat wave) events are predicted to increase in frequency and severity over the next decade (IPCC, 2013), and this future warming is expected to negatively impact the performance and survival of coastal organisms, because most organisms living in these areas already live close to their thermal tolerance limits (Hoegh-Guldberg et al., 2007). Although the present daily average summer temperatures in Tagus and Sado estuaries are well below the LT50 and CTMax of both clam species, the expected temperature increase (up to 4.8 °C; IPCC, 2013) will greatly reduce this thermal gap. Within the context of climate change and biological invasions, and in contrast to most studies on the subject, our study reveals that the alien-invasive *R. philippinarum* does not display significantly higher thermal tolerance limits than the native *R. decussatus*. Yet temperature had a stronger effect on metabolism and oxidative status of *R. decussatus*, as indicated by the higher metabolism (RMRs), HSR, lipid peroxidation (MDA) and antioxidant enzyme activities (GST, CAT and SOD). We argue that the alien-invasive species adopted the strategy of closing the valves during stressful short-term periods to ensure isolation from severe conditions and, consequently, guarantee longer survival. This behavioural strategy must entail aerobic metabolic depression and the shutdown of energetically expensive cellular processes. Thus, while it may confer ecophysiological advantages at present-day conditions, it will not be helpful under future chronic warming conditions. We argue that more comprehensive research is required to fully understand the greater success of alien-invasive species with the increase of global temperature at the biochemical/physiological level. Also, it is of paramount importance to evaluate other environmental stressors acting in synergy with temperature (namely hypoxia and acidification) that are also known to affect the fitness of coastal and estuarine communities.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2014.05.003.

Acknowledgements

The authors gratefully acknowledge Sandro Pinto and Pedro Patacas for their support in sample collection. This study was supported by the SHELLPLANT research project (ref. 232273) funded by the European Commission (FP7). Pest-C/EQB/LA0006/2013 was granted by Requimte. The first author acknowledges the PhD scholarship from the Portuguese Foundation for Science and Technology (FCT; ref. SFRH/BD/69274/2010), whereas the last two authors were supported by research contracts from the FCT Ciência 2007 (RR) and 2008 (AM) programs.

References

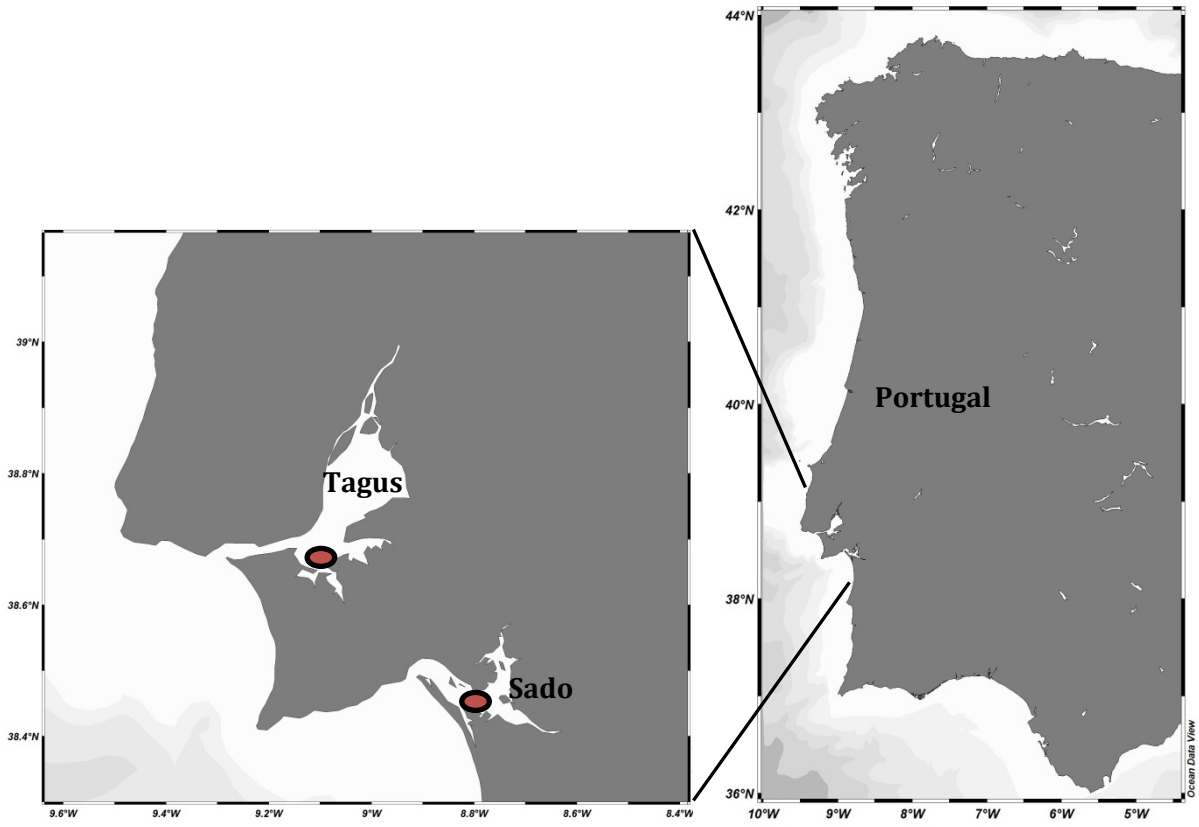
Abele, D., Puntarulo, S., 2004. Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 138 (4), 405–415.
Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S., 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. J. Exp. Biol. 205, 1831–1841.
Aebi, H.E., 1983. Catalase. In: Bergmeyer, H.U., Bergmeyer, J., Grassl, J.M. (Eds.), Methods of Enzymatic Analysis. VCH, Weinheim, Germany, pp. 273–286.
Anestis, A., Lazou, A., Pörtner, H.O., Michaelidis, B., 2007. Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293 (2), R911–R921.
Anestis, A., Pörtner, H.O., Karagiannis, D., Angelidis, P., Staikou, A., Michaelidis, B., 2010. Response of *Mytilus galloprovincialis* (L.) to increasing seawater temperature and to

marteliosis: metabolic and physiological parameters. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156 (1), 57–66.
Aurélio, M., Faleiro, F., Lopes, V.M., Pires, V., Lopes, A.R., Pimentel, M.S., Repolho, T., Baptista, M., Narciso, L., Rosa, R., 2013. Physiological and behavioral responses of temperate seahorses (*Hippocampus guttulatus*) to environmental warming. Mar. Biol. 160 (10), 2663–2670.
Bagnyukova, T.V., Lushchak, O.V., Storey, K.B., Lushchak, V.I., 2007. Oxidative stress and antioxidant defense responses by goldfish tissues to acute change of temperature from 3 to 23 °C. J. Therm. Biol. 32, 227–234.
Bolt, M.W., Mahoney, P.A., 1997. High-efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate-polyacrylamide, gel electrophoresis. Anal. Biochem. 247, 185–192.
Boutilier, R.G., 2001. Mechanisms of cell survival in hypoxia and hypothermia. J. Exp. Biol. 204, 3171–3181.
Braby, C.E., 2004. Physiological Ecology of Native and Invasive Blue Mussels (Genus *Mytilus*) in Central California. (PhD thesis) , Stanford University, California.
Braby, C.E., Somero, G.N., 2006. Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*). J. Exp. Biol. 209, 2554–2566.
Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Calosi, P., Bilton, D.T., Spicer, J.I., 2008. Thermal tolerance, acclimatory capacity and vulnerability to global climate change. Biol. Lett. 4, 99–102.
Capdevila-Argüelles, L., Zilletti, B., 2008. A perspective on climate change and invasive alien species. Convention on the Conservation of European Wildlife and Natural Habitats (T-PVS/Inf (2008) 5 rev, Strasbourg).
Childress, J.J., Seibel, B.A., 1998. Life at stable low oxygen levels: adaptations of animals to oceanic oxygen minimum layers. J. Exp. Biol. 201, 1223–1232.
Coccia, C., Calosi, P., Boyero, L., Green, A.J., Bilton, D.T., 2013. Does ecophysiology determine invasion success? A comparison between the invasive boatman *Trichocorixa verticalis verticalis* and the native *Sigara lateralis* (Hemiptera, Corixidae) in southwest Spain. PLoS One 8 (5), e63105.
Compton, T.J., Rijkenberg, M.J.A., Drent, J., Plersma, T., 2007. Thermal tolerance ranges and climate variability a comparison between bivalves from differing climates. J. Exp. Mar. Biol. Ecol. 352, 200–211.
De Zwaan, A., Thompson, R.J., Livingstone, D.R., 1980. Physiological and biochemical aspects of valve snap and valve closure in the giant scallop *Plactopecten magellanicus*. 2. Biochemistry. J. Comp. Physiol. 137, 105–115.
FAO, 2013. Species fact sheets: *Ruditapes decussatus* (Linnaeus, 1758). http://www.fao.org/fishery/culturedspecies/Ruditapes_decussatus/en (Accessed 26 August 2013).
Filho, D.W., 1996. Fish antioxidant defenses — a comparative approach. Braz. J. Med. Biol. Res. 29 (12), 1735–1742.
Flassch, J.P., Leborgne, Y., 1992. Introduction in Europe, from 1972 to 1980, of the Japanese Manila clam (*Tapes philippinarum*) and the effects on aquaculture production and natural settlement. ICES Mar. Sci. Symp. 194, 92–96.
Grieshaber, M.K., Hardewig, I., Kreutzer, U., Pörtner, H.O., 1994. Physiological and metabolic responses to hypoxia in invertebrates. Rev. Physiol. Biochem. Pharmacol. 125, 43–147.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic in mercapturic acid formation. J. Biol. Chem. 249 (22), 7130–7139.
Hall, D.M., Xu, L., Drake, V.J., Oberley, L.W., Oberley, T.D., Moseley, P.L., Kregel, K.C., 2000. Aging reduces adaptive capacity and stress protein expression in the liver after heat stress. J. Appl. Physiol. 89, 749–759.
Hardewig, I., Addink, A.D.F., Grieshaber, M.K., Pörtner, H.O., Van den Thillart, G., 1991. Metabolic rates at different oxygen levels determined by direct and indirect calorimetry in the oxyconformer *Sipunculus nudus*. J. Exp. Biol. 157, 143–160.
Hellmann, J.J., Byers, J.E., Bierwagen, B.G., Dukes, J.S., 2008. Five potential consequences of climate change for invasive species. Conserv. Biol. 22 (3), 534–543.
Helmuth, B., Mieszkowska, N., Moore, P., Hawkins, S.J., 2006. Living on the edge of two changing worlds: forecasting the responses of rocky intertidal ecosystems to climate change. Ann. Rev. Ecol. Evol. Syst. 37 (1), 373–404.
Henkel, S.K., Kawai, H., Hofmann, G.E., 2009. Interspecific and interhabitat variation in hsp70 gene expression in native and invasive kelp populations. Mar. Ecol. Prog. Ser. 386, 1–13.
Hochachka, P.W., Somero, G.N., 2002. Biochemical Adaptation: Mechanism and Process in Physiological Evolution. Oxford University Press, New York, USA.
Hoegh-Guldberg, O., Mumby, P.J., Hooten, A.J., Steneck, R.S., Greenfield, P., Gomez, E., Harvell, C.D., Sale, P.F., Edwards, A.J., Caldeira, K., Knowlton, N., Eakin, C.M., Iglesias-Prieto, R., Muthiga, N., Bradbury, R.H., Dubi, A., Hatziolos, M.E., 2007. Coral reefs under rapid climate change and ocean acidification. Science 318, 1737–1742.
Hofmann, G.E., Somero, G.N., 1996. Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination. Mar. Biol. 126 (1), 65–75.
IPCC, 2013. Climate change 2013: the physical science basis. In: Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.), Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA (1535 pp.).
Jansen, J.M., Hummel, H., Bonga, S.W., 2009. The respiratory capacity of marine mussels (*Mytilus galloprovincialis*) in relation to the high temperature threshold. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 153 (4), 399–402.
Jones, G.G., Sanford, C.L., Jones, B.L., 1993. Manila Clams: Hatchery and Nursery Methods. Innovative Aquaculture Products Ltd. and Science Council of British Columbia, Victoria, BC.
Jost, J.A., Podolski, S.M., Frederich, M., 2012. Enhancing thermal tolerance by eliminating the pejus range: a comparative study with three decapod crustaceans. Mar. Ecol. Prog. Ser. 444, 263–274.
Kondo, Y., 1987. Burrowing depth of infaunal bivalves—observation of living species and its relation to shell morphology. Trans. Proc. Palaeontol. Soc. Jpn (New Ser.) 148, 306–323.
Kregel, K.C., 2002. Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J. Appl. Physiol. 92 (5), 2177–2186.
Laruelle, F., Guillou, J., Paulet, Y.M., 1994. Reproductive pattern of the clams, *Ruditapes decussatus* and *R. philippinarum* on intertidal flats in Brittany. J. Mar. Biol. Assoc. U. K. 74, 351–366.
Lesser, M.P., 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annu. Rev. Physiol. 68, 253–278.
Li, Y., Schellhorn, H.E., 2007. Rapid kinetic microassay for catalase activity. J. Biomol. Tech. 18 (4), 185–187.
Lockwood, B.L., Somero, G.N., 2011. Invasive and native blue mussels (genus *Mytilus*) on the California coast: the role of physiology in a biological invasion. J. Exp. Mar. Biol. Ecol. 400, 167–174.
Lopes, A.R., Trübenbach, K., Teixeira, T., Lopes, V.M., Pires, V., Baptista, M., Repolho, T., Calado, R., Diniz, M., Rosa, R., 2013. Oxidative stress in deep scattering layers: heat shock response and antioxidant enzymes activities of myctophid fishes thriving in oxygen minimum zones. Deep-Sea Res., Part 1 Oceanogr. Res. Pap. 82, 10–16.
Lushchak, V., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101 (1), 13–30.
Mack, R.N., Simberloff, D., Lonsdale, W.M., Evans, H., Clout, M., Bazzaz, F.A., 2000. Biotic invasions: causes, epidemiology, global consequences and control. Ecol. Appl. 10, 689–710.
MacKenzie, B.R., Schiedek, D., 2007. Daily ocean monitoring since the 1860s shows record warming of northern European seas. Glob. Chang. Biol. 13, 1335–1347.
Mora, C., Ospína, A., 2001. Tolerance to high temperatures and potential impact of sea warming on reef fishes of Gorgona Island (tropical eastern Pacific). Mar. Biol. 139, 765–769.
Njemini, R., Demanet, C., Mets, T., 2005. Comparison of two ELISAs for the determination of Hsp70 in serum. J. Immunol. Methods 306 (1–2), 176–182.
Olyarnik, S.V., Bracken, M.E.S., Byrnes, J.E., Hughes, A.R., Hultgren, K.M., Stachowicz, J.J., 2009. Ecological factors affecting community invasibility. In: Rilov, G., Crooks, J.A. (Eds.), Biological Invasions in Marine Ecosystems: Ecological, Management, and Geographic Perspectives. Springer, Heidelberg, pp. 215–240.
Ortmann, C., Grieshaber, M.K., 2003. Energy metabolism and valve closure behaviour in the Asian clam *Corbicula fluminea*. J. Exp. Biol. 206, 4167–4178.
Otero, M., Cebrian, E., Francour, P., Galil, B., Savini, D., 2013. Monitoring Marine Invasive Species in Mediterranean Marine Protected Areas (MPAs): A Strategy and Practical Guide for Managers. IUCN, Malaga, Spain.
Øverli, Ø., Sørensen, C., Nilsson, G.E., 2006. Behavioral indicators of stress-coping style in rainbow trout: do males and females react differently to novelty? Physiol. Behav. 87, 506–512.
Pörtner, H.O., 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 132 (4), 739–761.
Pörtner, H.O., 2010. Oxygen and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J. Exp. Biol. 213, 881–893.
Pörtner, H.O., Knust, R., 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315, 95–97.
Pörtner, H.O., Mark, F.C., Bock, C., 2004. Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques. Respir. Physiol. Neurobiol. 141 (3), 243–260.
Rejmánek, M., 1995. What makes a species invasive? In: Pyšek, P., Prach, K., Rejmánek, M., Wade, M. (Eds.), Plant Invasions: General Aspects and Special Problems. SPB Academic Publishing, Amsterdam, pp. 3–13.
Rejmánek, M., 1996. A theory of seed plant invasiveness: the first sketch. Biol. Conserv. 78, 171–181.
Rejmánek, M., 2000. Invasive plants: approaches and predictions. Aust. Ecol. 25, 497–506.
Rejmánek, M., Richardson, D.M., 1996. What attributes make some plant species more invasive? Ecology 77, 1655–1661.
Rosa, R., Seibel, B.A., 2008. Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. Proc. Natl. Acad. Sci. U. S. A. 105, 20776–20780.
Rosa, R., Seibel, B.A., 2010. Voyage of the argonauts in the pelagic realm: physiological and behavioral ecology of the rare paper nautilus, *Argonauta nouryi*. ICES J. Mar. Sci. 67 (7), 1494–1500.
Rosa, R., Trueblood, L., Seibel, B.A., 2009. Ecophysiological influence on scaling of aerobic and anaerobic metabolism of pelagic gonatid squids. Physiol. Biochem. Zool. 82, 419–429.
Rosa, R., Pimentel, M.S., Boavida-Portugal, J., Teixeira, T., Trübenbach, K., Diniz, M.S., 2012. Ocean warming enhances malformations, premature hatching, metabolic suppression and oxidative stress in the early life stages of a keystone squid. PLoS One 7 (6), e38282.
Rosa, R., Trübenbach, K., Repolho, T., Pimentel, M., Faleiro, F., Boavida-Portugal, J., Baptista, M., Lopes, V.M., Dionísio, G., Leal, M.C., Calado, R., Pörtner, H.O., 2013. Lower hypoxia thresholds of cuttlefish early life stages living in a warm acidified ocean. Proc. R. Soc. B 280, 20131695.
Rosa, R., Trübenbach, K., Pimentel, M.S., Boavida-Portugal, J., Faleiro, F., Baptista, M., Calado, R., Pörtner, H.O., Repolho, T., 2014. Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). J. Exp. Biol. 217, 518–525.
Sokolova, I.M., Pörtner, H.O., 2001. Physiological adaptations to high intertidal life involve improved water conservation abilities and metabolic rate depression in *Littorina saxatilis*. Mar. Ecol. Prog. Ser. 224, 171–186.

Sol, D., Maspons, J., Vall-llosera, M., Bartomeus, I., García-Peña, G.E., Piñol, J., Freckleton, R.P., 2012. Unraveling the life history of successful invaders. Science 337, 580–583.
Somero, G.N., 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. J. Exp. Biol. 213, 912–920.
Sorte, C.J.B., Williams, S.L., Zerebecki, R.A., 2010. Ocean warming increases threat of invasive species in a marine fouling community. Ecology 91 (8), 2198–2204.
Sorte, C.J.B., Jones, S.J., Miller, L.P., 2011. Geographic variation in temperature tolerance as an indicator of potential population responses to climate change. J. Exp. Mar. Biol. Ecol. 400, 209–217.
Stachowicz, J.J., Terwin, J.R., Whitlatch, R.B., Osman, R.W., 2002. Linking climate change and biological invasions: ocean warming facilitates nonindigenous species invasions. Proc. Natl. Acad. Sci. U. S. A. 99, 15497–15500.
Stillman, J.H., Somero, G.N., 2000. A comparative analysis of the upper thermal tolerance limits of eastern pacific porcelain crabs, genus *Petrolisthes*: influences of latitude, vertical zonation, acclimation, and phylogeny. Physiol. Biochem. Zool. 73 (2), 200–208.
Streftaris, N., Zenetos, A., 2006. Alien marine species in the Mediterranean — the 100 'worst invasives' and their impact. Medit. Mar. Sci. 7, 87–118.
Sun, Y., Oberley, L.W., Li, Y., 1988. A simple method for clinical assay of superoxide dismutase. Clin. Chem. 34 (3), 497–500.
Taylor, A.C., 1976. Burrowing behaviour and anaerobiosis in the bivalve *Arctica islandica* (L.). J. Mar. Biol. Assoc. U. K. 56, 95–109.
Teixeira, T., Diniz, M., Calado, R., Rosa, R., 2013. Coral physiological adaptations to air exposure: heat shock and oxidative stress responses in *Veretillum cynomorium*. J. Exp. Mar. Biol. Ecol. 439, 35–41.
Tewksbury, J.J., Huey, R.B., Deutsch, C.A., 2008. Putting the heat on tropical animals. Science 320, 1296–1297.
Timofeyev, M.A., Shatilina, Z.M., Protopopova, M.V., Bedulina, D.S., Pavlichenko, V.V., Kolesnichenko, A.V., Steinberg, C.E.W., 2009. Thermal stress defense in freshwater amphipods from contrasting habitats with emphasis on small heat shock proteins (sHSPs). J. Therm. Biol. 34 (6), 281–285.
Tomanek, L., 2002. The heat-shock response: its variation, regulation and ecological importance in intertidal gastropods (genus *Tegula*). Integr. Comp. Biol. 42, 797–807.
Tomanek, L., 2008. The importance of physiological limits in determining biogeographical range shifts due to global climate change: the heat-shock response. Physiol. Biochem. Zool. 81 (6), 709–717.
Tomanek, L., 2010. Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. J. Exp. Biol. 213, 971–979.
Tomanek, L., Somero, G.N., 1999. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. J. Exp. Biol. 202, 2925–2936.
Uchiyama, M., Mihara, M., 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86 (1), 271–278.
Widdows, J., 1976. Physiological adaptation of *Mytilus edulis* to cyclic temperatures. J. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 105 (2), 115–128.
Zerebecki, Z.A., Sorte, C.J.B., 2011. Temperature tolerance and stress proteins as mechanisms of invasive species success. PLoS One 6 (4), e14806.

Supplementary data

Fig. S1. Map of the sampling area (red circles) in Tagus (*R. philippinarum*) and Sado (*R. decussatus*) estuaries.



CHAPTER 8

Effect of warming on protein, glycogen and fatty acid content of native and invasive clams

Anacleto P., Maulvault A.L., Bandarra N., Repolho T., Nunes M.L., Rosa R. and Marques A.

In press in *Food Research International*
DOI: 10.1016/j.foodres.2014.07.023

Effect of warming on protein, glycogen and fatty acid content of native and invasive clams

Patrícia Anacleto[a,b,c], Ana Luísa Maulvault[a,c], Narcisa M. Bandarra[a,c], Tiago Repolho[b], Maria Leonor Nunes[a,c], Rui Rosa[b], António Marques[a,c*]

[a] Division of Aquaculture and Upgrading (DivAV), Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.), Av. Brasília, 1449-006 Lisboa, Portugal
[b] Guia Marine Laboratory, Center of Oceanography, Faculty of Sciences, University of Lisbon (FCUL), Campo Grande, 1749-016 Lisboa, Portugal
[c] Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

ARTICLE INFO

Keywords:
Nutritional quality
Warming condition
Clams
Protein
Glycogen
Fatty acids

* Corresponding author.
Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.),
Avenida de Brasília, 1449–006, Lisboa, Portugal.
Tel.: +351 213 027 025; Fax: +351 21 3015948
E-mail address: amarques@ipma.pt (A. Marques)

ABSTRACT

Human bivalve consumption in Europe has steadily increased in the last years, particularly during summer months when seawater temperature increases. Since ocean warming is among the current global environmental threats affecting aquatic organisms, it is of paramount importance to investigate its effect on the nutritional quality of seafood products. In this context, the aim of this study was to investigate differences in the nutritional quality (in terms of protein, glycogen and fatty acid, FA, content) and condition of a native (grooved carpet shell, *Ruditapes decussatus*) and an invasive (Japanese carpet shell, *Ruditapes philippinarum*) clam species, subjected to warming. Our results clearly reveal that temperature significantly affected the nutritional quality of both clam species, particularly the FA composition. Both clam species responded similarly to warming, by significantly decreasing the content of some fatty acids, but not protein and glycogen levels. A predominance of polyunsaturated FA (PUFA) over saturated FA (SFA) and monounsaturated (MUFA) was observed throughout the experiment, as well as high *n*-3/*n*-6 and PUFA/SFA ratios. The native clam always revealed higher values of these fatty acids, indicating that this species has a better nutritional quality in comparison to the invasive one. Nonetheless, the loss of *n*-3 PUFA (in native species), eicosapentaenoic (EPA; in both species) and docosahexaenoic (DHA; in invasive species) acids was considered as the major negative outcome derived from warming, since it contributes to the loss of prime quality fatty acids for human health. However, atherogenic, thrombogenic and hypocholesterolemic/hypercholesterolemic indices (AI, TI and h/H, respectively) remained low in both species, even in warming conditions, suggesting that these food items can be used in a cardio-protective and hypocholesterolemic diet. This study provides new insights to understand and foretell the effects of climate change on nutritional quality of marine organisms.

1. Introduction

Worldwide, bivalve mollusc production has consistently increased in the last sixty years, ranging from approximately 1 million tons in 1950 to nearly 14.5 million tons in 2011, contributing 9.4% of the total amount of seafood produced in 2011, and the production is expected to increase in the future (FAO, 2014; OECD–FAO, 2013). Bivalve molluscs are considered as delicacies and healthy food items in several dietary regimes. Indeed, they provide high protein content, low energetic values, low fat/cholesterol contents, low proportion of saturated fat and high proportion of omega-3-fatty acids, essential amino acids, vitamin B_{12} and essential elements including iron, zinc and copper (Dong, 2001). Within bivalve molluscs, clams are the most successful species used for human consumption in Mediterranean countries, particularly grooved carpet shell clam (*Ruditapes decussatus*) and Japanese carpet shell clam (*Ruditapes philippinarum*) (Anacleto, Barrento, Nunes, Rosa, & Marques, 2014a).

Ruditapes decussatus is a native species in coastal lagoons and estuaries of the Atlantic and Mediterranean, while *R. philippinarum* is a native species from the Indo-Pacific region that was introduced in the beginning of the 1970's in Europe (North Atlantic and Mediterranean Sea; Flassch, & Leborgne, 1992). This species has been recognized among the most successful and worst invasive species in the Mediterranean, whose presence has a major impact both on biodiversity (e.g. competition with native species) and social-economic related activities (e.g. fisheries and aquaculture; Streftaris, & Zenetos, 2006).

Clam harvesting usually occurs in estuaries and coastal lagoons often subjected to strong anthropogenic pressures and climatic variability. Climate change is one of the greatest environmental challenges and is expected to worsen over the next decades. The average global sea surface temperatures are expected to increase 0.3–4.8 °C by the end of the 21st century (IPCC, 2013), leading to potentially negative impacts in seawater quality and consequently to marine organisms, since their metabolic processes are greatly influenced by temperature (FAO, 2008). Few studies have reviewed the potential effects of climate change on seafood chemical and biological safety (e.g. toxic metals, bacteria, virus, toxins from harmful algal blooms) and the implications for human health (Lake *et al.*, 2012; Marques, Nunes, Moore, & Strom, 2010; Tirado, Clarke, Jaykus, McQuatters-Gollop, & Frank, 2010). Despite several studies has been addressed the effects of increased temperature on physiological and cellular status of bivalves (e.g. Matoo, Ivanina, Ullstad, Beniash, & Sokolova, 2013; Matozzo *et al.*, 2012; Pernet, Tremblay, Comeau, & Guderley, 2007), there is only one study (Pernet *et al.*, 2007), to our knowledge, that have investigated the effect of this stressor on nutritional quality of bivalves at low temperatures, whereas this study addresses warming environments. Since clams are expected to be affected by ocean warming, the main goal of this study was to investigate differences in the nutritional quality (in terms of protein, glycogen and fatty acid content) of two bivalve molluscs subjected to warming conditions: a native (*R. decussatus*) and an invasive (*R. philippinarum*) clam species.

2. Materials and methods

2.1. Study area and collection of bivalves

Specimens of *R. decussatus* (total weight: 6.7 ± 1.1 g; edible weight: 3.0 ± 0.9 g; shell length: 31.2 ± 1.8 mm; shell width: 14.8 ± 0.9 mm; shell height: 22.5 ± 1.2 mm; mean ± standard deviation) and *R. philippinarum* (total weight: 13.2 ± 3.9 g; edible weight: 2.3 ± 0.4 g; shell length: 35.3 ± 3.0 mm; shell width: 18.8 ± 1.8 mm; shell height: 26.6 ± 2.3 mm; mean ± standard deviation) were hand collected within commercial fishing areas in the Sado and Tagus estuaries, respectively (Western coast of Portugal), during the summer season (August-September 2012). Additionally, sediment was collected from the same sampling sites. Afterwards, clam specimens were immediately transported in

portable insulated chambers with temperature controller (WAECO CoolFreeze CF-40, Emsdetten, Germany) to the laboratory and randomly placed in 14 flat-bottom cylindrical shaped fibreglass tanks (10 L capacity each; bottom-filled with 4 cm height of sampling sites sediment), within a recirculation aquaculture system (RAS), equipped with biological (ouriço®, Fernando Ribeiro Lda, Portugal), mechanical (100 µm, TMC-Iberia, Portugal) and physical filtration (ReefSkimPro 850, TMC-Iberia, Portugal). Thermo-regulation was provided by means of submerged water heaters (300W aquarium heater, Jager 3604, Eheim, GmbH & Co, Germany) and a chilling system (Frimar, Fernando Ribeiro Lda, Portugal). Seawater temperature, within the aquaculture system, was previously adjusted as to avoid thermal shock to collected specimens. Seawater used within the RAS was filtered (0.35 µm) and UV sterilized (Vecton 600, TMC-Iberia, Portugal). Ammonium and nitrite levels were determined daily by means of colorimetric test kits (Aquamerk, Merck Millipore, Germany) and kept below detectable levels. Salinity was daily checked and kept at 35 ± 1 (V2 refractometer, TMC, UK). Seawater was kept with continuous aeration (oxygen levels: 9.56 mg L^{-1}; WTW handheld Meter Multi 350i, Germany) and water temperature was maintained at 22.0 ± 0.2 °C, while pH was kept at 8.2 ± 0.1 and regulated by a CO_2 and soda lime filtered atmospheric air injection, controlled via a solenoid system, linked to an a automatic control system (Profilux 3.1N, GHL, Germany). Photoperiod was kept to 14 h light: 10 h dark. Acclimation temperature (22 °C) was chosen since it reflects the average thermal value that both clam species endure during the summer months in both estuaries. Clams were acclimated for 7 days and fed 3x day with a commercially available microalgae mix (1×10^5 cells/ml of *Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira* and *Nannochloropsis* spp.; Acuinuga, Coruña, Spain).

2.2. Survival

In order to determine the survival, organisms were placed into separated plastic containers, 20 specimens of each species, comprising three replicates (total n=60) and subjected to a water temperature gradient, within a thermostable water bath (Lauda, Lauda-Königshofen, Germany). Bath temperature started at the acclimation temperature (22 °C) and was maintained for 30 min. Thereafter, temperature was increased at a rate of 1 °C/1 hour and clams were observed continuously, until 38 °C (temperature at which clams began to die). In order to distinguish between live and dead specimens, inactive individuals were mechanically stimulated. The animals were considered as dead when valves were completely open and without any reaction to the stimulus. Dead specimens were removed and mortality was recorded. Seawater was continuously aerated (air stone atmospheric air injection) and temperature was continuously checked in each container using a digital thermometer (TFX 430, Ebro, Germany). At each temperature and for each species, four individuals were collected, immediately frozen in liquid nitrogen and stored at -80 °C. Since environmental variables that could influence results (e.g. oxygen, salinity, pH, feeding and temperature) were monitored during the acclimation and experiments, it was assumed that the observed results were due to temperature.

The frozen edible meat was recovered and adductor muscle of individual specimens was separated from mantles (including viscera) for posterior glycogen analysis. All tissues were freeze-dried separately for 48 h at -50 °C and low pressure (approximately 10^{-1} atm), followed by grinder homogenization, vacuum-packaging and storage at -80 °C until further analyses. Biochemical analyses were carried out in triplicate.

2.3. Protein analysis

Crude protein was analysed using the Dumas method (Saint-Denis, & Goupy, 2004), in a protein/nitrogen analyser (LECO FP-528, St Joseph, MI, USA) calibrated with EDTA (Cardoso, Mendes, Vaz-Pires, & Nunes, 2009). Analysis was performed in edible tissue of clams exposed at experimental temperatures

of 22, 28, 34 and 38 °C. Results were expressed as mg/g dry weight.

2.4. Glycogen analysis

Glycogen concentrations in bivalve samples were colorimetric determined using a solution of anthrone-sulfuric acid (Leyva, Quintana, Sánchez, Rodríguez, Cremata, & Sánchez, 2008; Viles, & Silverman, 1949). Briefly, adductor muscle samples (25 mg dry weight) were hydrolyzed with 15 mL of 33% potassium hydroxide (Panreac, Barcelona, Spain), in a water bath at 100 °C for 15 min. After sample cooling, 0.5 mL of hydrolized sample was mixed with 50 µL of a saturated sodium sulphate solution (Merck, Darmstadt, Germany) and 2 mL of 96% ethanol (AGA, Lisbon, Portugal). Samples were then placed in an ice bath for glycogen precipitation (~30 min) and centrifuged (14,000 *g*; 8 min), the precipitate was dissolved in 0.5 mL distilled water, precipitated again with 1 mL of ethanol (30 min) and centrifuged (14,000 *g*; 8 min). Additionally, the precipitate was once more dissolved in 0.5 mL of distilled water and 3 mL of anthrone-reagent, with resulting mixture heated to 90 °C for 20 min. The absorbance was measured at 620 nm (spectrophotometer UNICAM UV-Vis, Helios, United Kingdom). A calibration curve was prepared using glycogen (Sigma, USA) as standard. Anthrone-reagent was prepared with 38 mL concentrated sulphuric acid (Fluka, Riedel-de Haën, Germany), 15 mL distilled water and 0.075 g anthrone (Merck, Darmstadt, Germany). Glycogen content was calculated from 22 to 38 °C (every two Celsius degrees) and results were expressed as mg/g dry weight.

2.5. Fatty acid analysis

Fatty acids methyl esters (FAME) of total lipids were prepared through acid-catalyzed transesterification (Cohen, Vonshak, & Richmond, 1988). Each sample, containing approximately 100 mg of edible tissue dry weight, was dissolved in 5 mL acetyl chloride:methanol (1:19, v/v; Merck), shaken and heated (80 °C; 1 h). After cooling, 1 mL Milli-Q distilled water and 2 mL n-heptane (99.5%; Merck) were added to each sample. Afterwards, samples were shaken and centrifuged (2300 *g*; 5 min; Sigma 3K30, Germany) to enable phase separation. Consequently, the moisture content of the upper phase was removed with anhydrous sodium sulphate (99.0%; Panreac). Subsequently, an aliquot (2 µL) of the upper phase was then injected onto a gas chromatograph (Varian Star 3800 Cp, Walnut Creek, CA, USA), equipped with an auto-sampler and fitted with a flame ionisation detector at 250 °C, in order to perform FAME analysis. The separation of fatty acids was performed in a DBWax polyethylene glycol column (30 m length × 0.32 mm internal diameter; 0.25 µm film thickness; Hewlett-Packard, Albertville, MN, USA) and helium was used as carrier gas at a flow rate of 1 mL min-1, programmed to start at 180 °C, held at this temperature for 5 min, raised to 220 °C at 4 °C min^{-1}, and maintained at 220 °C for 25 min, with the detector and the split injector (100:1) at 250 °C. Fatty acids methyl esters were identified by retention time comparison with those of Sigma–Aldrich standards (St. Louis, MA, USA). Quantitative data were calculated using the peak area ratio (% of total fatty acids; FA) and the Varian software (Varian Star 3800 Cp). A volume of 25 µL of a solution of heneicosanoic acid (5 mg mL^{-1}; Sigma–Aldrich, C21:0) in n-heptane (Merck, 99.5%) was used as an internal standard (IS) to convert the percentage of each fatty acid (FA) into mg/g:

$$FA = \frac{FA\ area\ \times IS\ mass\ (mg)}{IS\ area\ \times sample\ mass\ (g)}$$

Total FA was converted into total lipids by dividing by a factor of 0.7, which was previously described for lean species such as bivalve molluscs (Weihrauch, Posati, Anderson, & Exler, 1977). This analysis was only performed at 22, 28, 34 and 38 °C and the results were expressed as mg/g dry weight.

2.5.1. Nutritional quality

The atherogenic and thrombogenic indices are important tools, in a consumer point of view, to estimate the probability of

developing coronary heart diseases, being calculated for each clam species according to the Ulbricht, and Southgate (1991) equations: AI = [12:0 + (4 × 14:0) + 16:0]/[ΣMUFA + ΣPUFA (*n*-6) + (*n*-3)]; TI=(14:0 + 16:0 + 18:0)/[(0.5 × ΣMUFA) + (0.5 × ΣPUFA (*n*-6)) + (3 × ΣPUFA (*n*-3) + (*n*-3)/*n*-6))]. The hypocholesterolemic/hypercholesterolemic index of the clams' species was evaluated according to Santos-Silva, Bessa, and Santos-Silva (2002) equation: h/H = (18:1*n*-9 + 18:2*n*-6 + 20:4*n*-6 + 18:3*n*- 3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3)/(14:0 + 16:0). Two ratios related with fatty acid content were also calculated: DHA/EPA (docosahexaenoic acid/eicosapentaenoic acid) and PUFA/SFA (polyunsaturated fatty acid/saturated fatty acid); as well as monounsaturated (MUFA), omega-3 and omega-6 fatty acids.

2.6. Statistical analyses

Data were analysed for normality and variance homogeneity through Kolmogorov–Smirnov and Levene's tests, respectively. One-way analysis of variance (ANOVA) or non-parametric testing (Kruskal–Wallis) was performed as to detect significant statistical differences. Subsequently, post-hoc tests (Tukey HSD and unequal N HSD) or non-parametric multiple comparison test (Dunn´s) were performed. Additionally, two-way ANOVA was performed to identify statistically significant differences in analysed parameters (protein, glycogen and principal fatty acids contents) between clam species and temperature. Statistical analysis was performed for a significance level of 0.05, using STATISTICA™ software (Version 7.0, StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results and discussion

No mortality was registered for the invasive species between 22 and 38 °C, as well as the native species between 22 and 36 °C, but attaining 5% mortality at 38 °C. This result is in accordance with those obtained by Anacleto *et al.* (2014b), where the invasive clam species was shown to have greater physiological plasticity under stress conditions than the native one. In this way, *R. philippinarum* can tolerate habitats subjected to greater biotic and abiotic environmental variations and have greater distribution range, which is the key for a species invasion success (Olyarnik *et al.*, 2009). Previous studies confirm the higher tolerance of invasive species to environmental temperature variations compared to native species, such as the bivalve species *Mytilus galloprovincialis* (Lockwood, & Somero, 2011), *Brachidontes exustus*, *Perna viridis*, *P. perna*, *Isognomon bicolor*, *Saccostrea glomerata* and *Crassostrea gigas* (Lenz *et al.*, 2011), and marine fouling bryozoans and tunicates (Sorte, Williams, & Zerebecki, 2010).

Concerning biochemical parameters, proteins reached high values in both clam species, with significantly higher values being always registered in native species. Proteins have an important physiological role in the supply of structural elements and in the catalysis of metabolic reactions (Dittrich, 1991). Warming conditions did not significantly affect protein levels in both species, except at 34 °C in the invasive species, where a significant increase was registered compared to 22 °C ($p=0.023$; Fig. 1A), likely due to intraspecific variations. Indeed, many authors highlighted that the use of the protein fraction as an energy source for the maintenance of bivalve metabolic needs only takes place when carbohydrate and lipid reserves have already been greatly depleted (Barber, & Blake, 1981). Although only invasive species showed low glycogen levels at higher temperatures (after 36 °C; Fig. 1B), this species appears not use proteins as energy to support these extreme temperatures. Thus, the authors argue that proteins may not have been used to supply energy during thermal stress in both clam species.

Glycogen is the main carbohydrate reserve in bivalves, and has been reported that under stressful conditions glycogen is primarily used for the maintenance of animal condition (Bayne, Thompson, & Widdows, 1976; Patrick, Faury, & Goulletquer, 2006). In this study, the native species *R. decussatus* always showed significantly higher glycogen levels than the invasive species (Fig. 1B).

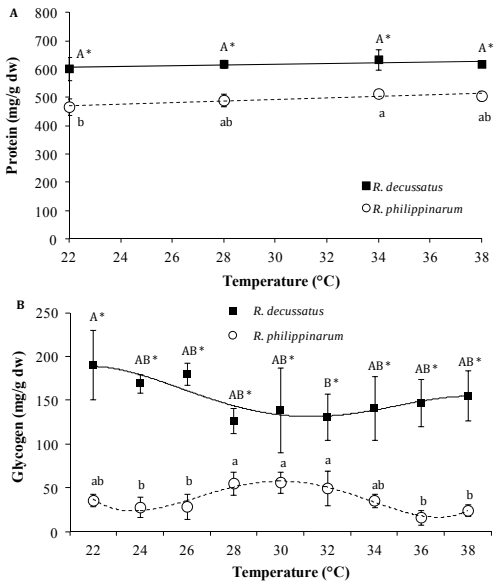


Fig. 1. Effect of warming on A) protein and B) glycogen contents (mg/g dw) of the native clam (*R. decussatus*) and the invasive clam (*R. philippinarum*). Values represent mean ± SD (n=4 per each temperature). Black and dashed lines represent trendlines for each species. Different letters (capital letters for *R. decussatus*; small letters for *R. philippinarum*) represent significant differences ($p<0.05$) between temperatures, and asterisks represent significant differences between species at each temperature.

Glycogen levels only significantly decreased with temperature gradient in the native species (at 32 °C compared to 22 °C), but no differences were registered for the invasive species. Hence, the current study reveals that only the native species seems to use this reserve to combat thermal stress.

Fatty acids are the fundamental structural components of almost all forms of lipids, acting as precursors of bioactive molecules (e.g., prostaglandins) and have structural and functional role in organisms, which affects processes such a reproduction, osmoregulation and stress response (Makoto *et al.*, 1989). In this study, the native species revealed valuable PUFA content (17.72 mg/g dw), followed by SFA and MUFA (13.75 and 9.40 mg/g dw, respectively), while in the invasive species also showed higher PUFA (12.05 mg/g dw) values followed by similar SFA and MUFA contents (5.92 and 5.81 mg/g dw, respectively; Table 1). The fatty acids levels in both clam species were similar to those found in previous studies (Bandarra *et al.*, 2004). The main SFA were palmitic acid (PA; 16:0) and stearic acid (SA; 18:0) in both species (Table 1). PA is the major end-product of the fatty acid synthesis in animal tissues and the precursor for de novo synthesis of long-chain saturated and unsaturated fatty acids (Gabbott, 1983). Among MUFA, vaccenic acid (VA; 18:1*n*-7) was the prevailing fatty acid in the native species, contrasting to gadoleic acid (GA; 20:1*n*-11) in the invasive species, followed by oleic acid (OA; 18:1*n*-9) and erucic acid (EA; 22:1*n*-9) in both species (Table 1). The main *n*-3 PUFA were eicosapentaenoic (EPA; 20:5*n*-3) and docosahexaenoic (DHA; 22:6*n*-3) acids in both species (Table 1), which are essential nutrients for marine invertebrates (Sargent, Bell, McEvoy, Tocher, & Estevez, 1999). In invasive species, hexadecatrienoic acid (HTA; 16:3*n*-3) was also an important fatty acid. Arachidonic acid (AA; 20:4*n*-6) was the most prevalent *n*-6 PUFA in both clams (Table 1). It is noteworthy that both clams had generally very low total lipid content which is usually around 1.0 g per 100g wet weight in *R. decussatus* (Gonçalves, Pedro, Duarte, & Nunes, 2009) and 0.75 g per 100g wet weight in *R. philippinarum* (Oliveira, 2012). Since both clam species were fed with the same diet throughout the acclimation period, the differences found in fatty acid content might be related to distinct metabolic rates and clearance rates, as suggested by Pernet *et al.* (2007) for mussels and oysters and/or differences in the gametogenic status (Soudant *et al.*, 1999). In warming conditions, the native species still revealed significantly higher levels of most fatty acids than the invasive species (Table 1), except for arachidonic acid (AA; 20:4*n*-6) and PUFA/SFA ratio. Prevalence of PUFA over SFA and MUFA was also observed in warming conditions, for both clam species, though the highest levels were found at lower temperatures (22 °C; Table 1). This result indicates that both clam species use fatty acids as an energy source during thermal stress.

Temperature strongly affected fatty acid profile in both clam species (Table 1). The majority of fatty acids significantly decreased with warming water in: a) native species after 22 °C for SFA (mainly due to PA and SA),

Table 1. Fatty acid quantitative composition (mg/g dw) of the native (*Ruditapes decussatus*) and invasive clams (*Ruditapes philippinarum*) at different temperatures.

	Ruditapes decussatus				*Ruditapes philippinarum*				Significant effect
Fatty acids	**22 °C**	**28 °C**	**34 °C**	**38 °C**	**22 ºC**	**28 ºC**	**34 ºC**	**38 ºC**	
SFA									
14:0	0.14 ± 0.03^{a}	0.16 ± 0.01^{a}	0.13 ± 0.01^{a}	0.17 ± 0.04^{a}	0.09 ± 0.01^{a}	0.10 ± 0.02^{a}	0.11 ± 0.02^{a}	0.11 ± 0.02^{a}	S
Iso 15:0	0.05 ± 0.02^{a}	0.02 ± 0.00^{b}	0.03 ± 0.00^{ab}	0.04 ± 0.01^{ab}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}	S, T×S
15:0	0.19 ± 0.05^{a}	0.09 ± 0.01^{b}	0.10 ± 0.01^{b}	0.12 ± 0.01^{b}	0.07 ± 0.01^{a}	0.08 ± 0.02^{a}	0.08 ± 0.02^{a}	0.07 ± 0.01^{a}	T, S, T×S
Anteiso 16:0	0.07 ± 0.02^{a}	0.08 ± 0.00^{a}	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}	0.05 ± 0.01^{a}	0.04 ± 0.00^{a}	0.06 ± 0.01^{a}	0.04 ± 0.01^{a}	S, T×S
16:0	7.77 ± 1.12^{a}	4.76 ± 0.87^{b}	4.85 ± 1.13^{b}	4.93 ± 0.13^{b}	2.72 ± 0.32^{a}	2.68 ± 0.24^{a}	2.78 ± 0.29^{a}	2.76 ± 0.44^{a}	T, S, T×S
Iso 17:0	0.68 ± 0.15^{a}	0.50 ± 0.04^{a}	0.49 ± 0.05^{a}	0.55 ± 0.06^{a}	0.37 ± 0.07^{a}	0.33 ± 0.03^{a}	0.34 ± 0.02^{a}	0.30 ± 0.05^{a}	S
17:0	0.51 ± 0.04^{a}	0.43 ± 0.03^{a}	0.43 ± 0.05^{a}	0.48 ± 0.02^{a}	0.32 ± 0.04^{a}	0.27 ± 0.02^{a}	0.29 ± 0.01^{a}	0.25 ± 0.04^{a}	T, S, T×S
18:0	2.67 ± 0.05^{a}	2.10 ± 0.22^{b}	2.37 ± 0.22^{ab}	2.22 ± 0.03^{ab}	1.88 ± 0.11^{a}	1.49 ± 0.10^{b}	1.61 ± 0.17^{ab}	1.63 ± 0.16^{ab}	T, S
19:0	0.10 ± 0.02^{a}	0.06 ± 0.00^{b}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}	0.05 ± 0.00^{ab}	0.05 ± 0.00^{ab}	0.05 ± 0.01^{a}	0.04 ± 0.01^{b}	T, S, T×S
20:0	0.11 ± 0.01^{a}	0.06 ± 0.00^{b}	0.07 ± 0.01^{b}	0.07 ± 0.01^{b}	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}	0.07 ± 0.00^{a}	0.05 ± 0.01^{a}	T, S, T×S
22:0	0.03 ± 0.00^{a}	0.05 ± 0.00^{a}	0.04 ± 0.00^{a}	0.03 ± 0.00^{a}	0.07 ± 0.01^{a}	0.04 ± 0.01^{a}	0.07 ± 0.01^{a}	0.04 ± 0.01^{a}	S
∑ SFA	13.75 ± 1.06^{a}	7.81 ± 0.49^{b}	9.11 ± 1.12^{b}	8.77 ± 0.23^{b}	5.92 ± 0.19^{a}	5.11 ± 0.40^{b}	5.47 ± 0.39^{ab}	5.52 ± 0.55^{ab}	T, S, T×S
MUFA									
16:1*n*-9	0.59 ± 0.08^{a}	0.72 ± 0.05^{a}	0.62 ± 0.15^{a}	0.60 ± 0.04^{a}	0.45 ± 0.04^{a}	0.43 ± 0.02^{a}	0.38 ± 0.04^{ab}	0.34 ± 0.02^{b}	S
16:1*n*-7	0.64 ± 0.20^{a}	0.48 ± 0.11^{a}	0.49 ± 0.11^{a}	0.53 ± 0.11^{a}	0.29 ± 0.05^{a}	0.27 ± 0.06^{a}	0.30 ± 0.07^{a}	0.37 ± 0.03^{a}	S
16:1*n*-5	0.07 ± 0.02^{b}	0.15 ± 0.02^{a}	0.05 ± 0.02^{b}	0.14 ± 0.02^{a}	0.07 ± 0.01^{a}	0.02 ± 0.00^{b}	0.06 ± 0.00^{ab}	0.05 ± 0.02^{ab}	T, S, T×S
17:1	0.13 ± 0.02^{a}	0.09 ± 0.01^{b}	0.10 ± 0.02^{ab}	0.09 ± 0.02^{ab}	0.06 ± 0.02^{a}	0.05 ± 0.01^{a}	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	T, S
17:1*n*-8	0.48 ± 0.04^{a}	0.42 ± 0.03^{a}	0.45 ± 0.07^{a}	0.43 ± 0.03^{a}	0.24 ± 0.03^{a}	0.18 ± 0.02^{ab}	0.19 ± 0.03^{ab}	0.15 ± 0.02^{b}	T, S
18:1*n*-11	0.09 ± 0.02^{a}	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}	0.09 ± 0.03^{a}	0.06 ± 0.01^{a}	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}	0.05 ± 0.01^{a}	T, S
18:1*n*-9	1.36 ± 0.15^{a}	0.79 ± 0.12^{b}	0.80 ± 0.21^{b}	0.88 ± 0.11^{b}	0.95 ± 0.10^{a}	0.96 ± 0.04^{a}	0.89 ± 0.10^{a}	0.73 ± 0.06^{a}	T, T×S
18:1*n*-7	1.66 ± 0.12^{a}	0.75 ± 0.10^{b}	0.85 ± 0.20^{b}	0.92 ± 0.21^{b}	0.42 ± 0.04^{a}	0.40 ± 0.05^{a}	0.38 ± 0.06^{a}	0.39 ± 0.06^{a}	T, S, T×S
20:1*n*-11	0.71 ± 0.24^{a}	0.68 ± 0.07^{a}	0.62 ± 0.11^{a}	0.67 ± 0.04^{a}	1.09 ± 0.06^{a}	0.90 ± 0.04^{b}	1.01 ± 0.05^{a}	0.88 ± 0.03^{b}	S
20:1*n*-9	0.37 ± 0.03^{a}	0.32 ± 0.07^{a}	0.31 ± 0.08^{a}	0.33 ± 0.05^{a}	0.41 ± 0.04^{a}	0.41 ± 0.05^{a}	0.39 ± 0.03^{a}	0.39 ± 0.08^{a}	S
20:1*n*-7	0.59 ± 0.09^{a}	0.54 ± 0.01^{a}	0.62 ± 0.03^{a}	0.66 ± 0.06^{a}	0.30 ± 0.04^{a}	0.25 ± 0.02^{ab}	0.27 ± 0.01^{ab}	0.23 ± 0.04^{b}	S, T×S
22:1*n*-11	0.61 ± 0.04^{a}	0.53 ± 0.02^{a}	0.52 ± 0.08^{a}	0.53 ± 0.09^{a}	0.69 ± 0.06^{a}	0.63 ± 0.04^{ab}	0.64 ± 0.04^{ab}	0.54 ± 0.08^{b}	T, S
22:1*n*-9	1.35 ± 0.06^{a}	1.42 ± 0.11^{a}	1.23 ± 0.20^{a}	1.34 ± 0.11^{a}	0.71 ± 0.03^{a}	0.68 ± 0.03^{ab}	0.59 ± 0.07^{bc}	0.57 ± 0.03^{c}	S
∑ MUFA	9.40 ± 0.19^{a}	6.94 ± 0.29^{b}	7.35 ± 0.22^{b}	7.43 ± 0.58^{b}	5.81 ± 0.32^{a}	5.37 ± 0.18^{ab}	5.37 ± 0.31^{ab}	4.97 ± 0.58^{b}	T, S, T×S
PUFA									
16:2*n*-4	0.12 ± 0.01^{a}	0.07 ± 0.01^{a}	0.09 ± 0.02^{a}	0.10 ± 0.04^{a}	0.15 ± 0.02^{a}	0.14 ± 0.02^{a}	0.15 ± 0.02^{a}	0.09 ± 0.03^{b}	T, S, T×S
16:3*n*-4	0.45 ± 0.08^{a}	0.38 ± 0.04^{a}	0.37 ± 0.04^{a}	0.47 ± 0.11^{a}	0.39 ± 0.04^{a}	0.32 ± 0.02^{ab}	0.33 ± 0.02^{ab}	0.27 ± 0.05^{b}	S
16:3*n*-3	2.51 ± 0.10^{a}	2.03 ± 0.14^{b}	2.30 ± 0.14^{ab}	1.97 ± 0.20^{b}	1.95 ± 0.11^{a}	1.90 ± 0.11^{a}	1.68 ± 0.15^{ab}	1.50 ± 0.05^{b}	T, S, T×S

Table 1. (continued)

	Ruditapes decussatus				*Ruditapes philippinarum*				Significant effect
Fatty acids	**22 °C**	**28 °C**	**34 °C**	**38 °C**	**22 ºC**	**28 ºC**	**34 ºC**	**38 ºC**	
18:2*n*-6	0.31 ± 0.07^{a}	0.10 ± 0.02^{b}	0.11 ± 0.02^{b}	0.11 ± 0.03^{b}	0.25 ± 0.03^{a}	0.26 ± 0.02^{a}	0.27 ± 0.06^{a}	0.19 ± 0.02^{a}	T, S, T×S
18:3*n*-6	0.10 ± 0.03^{a}	0.04 ± 0.00^{a}	0.05 ± 0.02^{a}	0.06 ± 0.02^{a}	0.04 ± 0.00^{a}	0.03 ± 0.01^{a}	0.04 ± 0.00^{a}	0.03 ± 0.00^{a}	T, S, T×S
18:3*n*-4	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}	0.03 ± 0.00^{a}	0.05 ± 0.00^{a}	0.05 ± 0.01^{a}	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	S
18:3*n*-3	0.22 ± 0.02^{a}	0.06 ± 0.02^{c}	0.11 ± 0.01^{b}	0.06 ± 0.00^{bc}	0.12 ± 0.02^{a}	0.11 ± 0.03^{a}	0.07 ± 0.01^{a}	0.10 ± 0.03^{a}	T, S, T×S
18:4*n*-3	0.40 ± 0.03^{a}	0.13 ± 0.02^{b}	0.12 ± 0.02^{b}	0.09 ± 0.00^{b}	0.13 ± 0.01^{a}	0.12 ± 0.03^{a}	0.08 ± 0.03^{a}	0.09 ± 0.03^{a}	T, S, T×S
20:2*n*-9	0.16 ± 0.06^{a}	0.13 ± 0.04^{a}	0.13 ± 0.04^{a}	0.13 ± 0.00^{a}	0.11 ± 0.03^{a}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}	0.06 ± 0.01^{b}	T, S, T×S
20:2*n*-6	0.68 ± 0.06^{a}	0.61 ± 0.08^{a}	0.50 ± 0.11^{a}	0.77 ± 0.18^{a}	0.68 ± 0.06^{a}	0.56 ± 0.05^{ab}	0.57 ± 0.05^{ab}	0.46 ± 0.08^{b}	T×S
20:4*n*-6	0.97 ± 0.06^{a}	1.04 ± 0.11^{a}	1.01 ± 0.07^{a}	0.96 ± 0.07^{a}	1.07 ± 0.05^{a}	1.05 ± 0.08^{a}	1.01 ± 0.03^{a}	0.85 ± 0.09^{b}	T
20:4*n*-3	0.13 ± 0.02^{a}	0.12 ± 0.02^{a}	0.11 ± 0.01^{a}	0.12 ± 0.03^{a}	0.06 ± 0.01^{a}	0.07 ± 0.02^{a}	0.04 ± 0.01^{a}	0.05 ± 0.02^{a}	S
20:5*n*-3 (EPA)	3.43 ± 0.20^{a}	2.00 ± 0.11^{b}	2.25 ± 0.14^{b}	2.45 ± 0.15^{b}	1.86 ± 0.20^{a}	1.74 ± 0.13^{a}	1.87 ± 0.16^{a}	1.04 ± 0.28^{b}	T, S, T×S
21:5*n*-3	0.16 ± 0.05^{a}	0.10 ± 0.00^{a}	0.11 ± 0.02^{a}	0.16 ± 0.01^{a}	0.12 ± 0.02^{a}	0.11 ± 0.03^{a}	0.10 ± 0.02^{a}	0.10 ± 0.02^{a}	NS
22:3*n*-9	0.31 ± 0.02^{b}	0.43 ± 0.05^{a}	0.31 ± 0.04^{b}	0.42 ± 0.04^{a}	0.25 ± 0.02^{b}	0.37 ± 0.02^{a}	0.25 ± 0.02^{b}	0.30 ± 0.05^{ab}	T, S
22:3*n*-6	0.31 ± 0.01^{b}	0.40 ± 0.03^{a}	0.29 ± 0.04^{b}	0.33 ± 0.02^{b}	0.27 ± 0.02^{a}	0.26 ± 0.04^{a}	0.24 ± 0.02^{a}	0.22 ± 0.05^{a}	T, S, T×S
22:4*n*-6	0.27 ± 0.03^{a}	0.34 ± 0.04^{a}	0.36 ± 0.07^{a}	0.37 ± 0.04^{a}	0.37 ± 0.02^{a}	0.34 ± 0.02^{a}	0.34 ± 0.05^{a}	0.31 ± 0.04^{a}	T×S
22:5*n*-6	0.21 ± 0.02^{a}	0.23 ± 0.01^{a}	0.20 ± 0.03^{a}	0.22 ± 0.01^{a}	0.40 ± 0.02^{a}	0.36 ± 0.03^{ab}	0.35 ± 0.02^{ab}	0.32 ± 0.02^{b}	S, T×S
22:5*n*-3	0.87 ± 0.10^{a}	0.68 ± 0.08^{a}	0.72 ± 0.04^{a}	0.71 ± 0.07^{a}	0.42 ± 0.03^{a}	0.37 ± 0.03^{a}	0.38 ± 0.02^{a}	0.36 ± 0.05^{a}	T, S
22:6n-3 (DHA)	6.22 ± 0.58a	5.81 ± 0.66a	5.63 ± 1.00a	6.28 ± 0.17a	3.05 ± 0.21^{a}	2.83 ± 0.12^{ab}	2.68 ± 0.32^{ab}	2.39 ± 0.24^{b}	S
∑ PUFA	17.72 ± 0.66^{a}	14.61 ± 0.74^{b}	15.24 ± 1.03^{b}	15.50 ± 1.30^{b}	12.05 ± 0.78^{a}	10.27 ± 0.28^{ab}	10.11 ± 0.76^{b}	8.85 ± 0.88^{b}	T, S, T×S
∑PUFA*n*-3	13.71 ± 0.74^{a}	10.80 ± 0.48^{b}	11.58 ± 1.15^{b}	11.67 ± 1.21^{b}	7.29 ± 0.56^{a}	6.96 ± 0.54^{a}	7.64 ± 0.79^{a}	6.78 ± 1.03^{a}	T, S, T×S
∑PUFA*n*-6	3.39 ± 0.12^{a}	2.67 ± 0.13^{b}	2.75 ± 0.09^{b}	2.78 ± 0.26^{b}	3.06 ± 0.13^{a}	2.82 ± 0.21^{a}	2.87 ± 0.03^{a}	2.35 ± 0.13^{b}	T, T×S
∑n-3/∑*n*-6	5.52 ± 0.87^{a}	4.04 ± 0.13^{b}	4.66 ± 0.59^{ab}	4.42 ± 0.31^{ab}	2.50 ± 0.22^{a}	2.54 ± 0.18^{a}	2.24 ± 0.41^{a}	2.47 ± 0.22^{a}	T, S, T×S
∑*n*-6/∑*n*-3	0.20 ± 0.04^{a}	0.23 ± 0.03^{a}	0.22 ± 0.03^{a}	0.23 ± 0.03^{a}	0.40 ± 0.03^{a}	0.40 ± 0.03^{a}	0.34 ± 0.05^{a}	0.41 ± 0.04^{a}	S
DHA/EPA	3.69 ± 0.94^{a}	3.18 ± 0.76^{a}	3.54 ± 0.54^{a}	3.09 ± 0.67^{a}	1.99 ± 0.14^{a}	1.86 ± 0.36^{a}	1.81 ± 0.37^{a}	1.63 ± 0.29^{a}	S
PUFA/SFA	1.53 ± 0.23^{a}	1.87 ± 0.02^{a}	1.79 ± 0.11^{a}	1.63 ± 0.25^{a}	1.99 ± 0.10^{a}	2.08 ± 0.08^{a}	1.85 ± 0.09^{a}	2.00 ± 0.10^{a}	S, T×S
AI	0.32 ± 0.02^{a}	0.25 ± 0.02^{b}	0.26 ± 0.03^{b}	0.26 ± 0.01^{b}	0.21 ± 0.02^{a}	0.20 ± 0.01^{a}	0.23 ± 0.03^{a}	0.21 ± 0.03^{a}	T, S, T×S
TI	0.20 ± 0.03^{a}	0.16 ± 0.00^{a}	0.16 ± 0.01^{a}	0.18 ± 0.03^{a}	0.16 ± 0.01^{a}	0.16 ± 0.01^{a}	0.17 ± 0.00^{a}	0.16 ± 0.01^{a}	S
h/H	1.79 ± 0.36^{a}	2.29 ± 0.12^{a}	2.21 ± 0.22^{a}	1.78 ± 0.48^{a}	2.51 ± 0.12^{a}	2.52 ± 0.17^{a}	2.31 ± 0.25^{a}	2.49 ± 0.09^{a}	S, T×S
∑ Total FA	37.40 ± 2.91^{a}	30.64 ± 2.81^{b}	30.94 ± 0.82^{b}	31.69 ± 2.11^{b}	24.08 ± 1.63^{a}	20.91 ± 1.41^{ab}	20.80 ± 0.98^{ab}	17.76 ± 1.84^{b}	T, S
Total lipids	53.42 ± 4.15^{a}	43.77 ± 4.02^{b}	44.20 ± 1.18^{b}	45.28 ± 3.01^{b}	34.40 ± 2.32^{a}	29.87 ± 2.02^{ab}	29.72 ± 1.40^{ab}	25.37 ± 2.63^{b}	T, S

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AI, atherogenic index; TI, thrombogenic index; h/H, hypocholesterolemic/hypercholesterolemic index; FA, total fatty acid; T, temperature; S, species; NS, not significant. Values are means (±SD, n=4). Different superscript letters represent significant differences ($p<0.05$) between temperatures (a-c). *calculated with a corrective factor of 0.7.

MUFA (due to OA and VA), PUFA (due to linoleic acid, LA, 18:2*n*-6 and EPA), *n*-3 and *n*-6 PUFAs, *n*-3/*n*-6 ratio and total FA; and b) invasive species after 22 °C for SFA (due to SA), after 28 °C for PUFA, and after 34 °C for MUFA, AA, EPA, DHA and total FA. It is also important to note that relevant levels of 20:1 and 22:1 were observed in both clam species, and did not decrease with warming, particularly in native species, since these fatty acids are generally from exogenous origin (e.g. available feed; Bandarra, Batista, & Nunes, 2009). Many studies with marine and terrestrial invertebrates corroborate the lower levels in some MUFA (e.g. OA, VA) and/or PUFA (e.g. EPA) at higher temperatures (20 °C) compared to 0 °C (e.g. Pernet et al., 2007; Van Dooremalen, Koekkoek, & Ellers, 2011). This is in accordance with the fact that some fatty acids (e.g. PUFA) are essential to maintain the fluidity and permeability of cell membranes of ectotherms (homeoviscous adaptation, HVA) to temperature fluctuations. Indeed, the saturation of membrane fatty acids is increased during a warm response to compensate the negative effect of membrane fluidity, i.e. as temperature is raised acutely, fluidity is increased beyond the optimal range and the membrane becomes "hyperfluid" (Hochachka, & Somero, 2002). These changes are commonly induced by the activation of acyltransferases, phosphoglyceride transferases and fatty acid desaturases (Kayama, Hirata, & Hisai, 1986). However, Van Dooremalen *et al.* (2011) also observed a decrease in the main SFA (PA and SA) with increasing temperature in insects, which is against to the expected response because of its solidifying effects on membrane fluidity. Such decrease in SFA might be related to the metabolic needs (e.g. use as energy source) of bivalves in extreme warming conditions. However, above 20 °C the trend is unknown, but seems to behave in a similar way.

The variations in fatty acid composition induced by warming might also have other explanations. Indeed, it is known that PUFA are easy targets for reactive oxygen species (ROS) driven oxidation, and once the process of lipid radical formation is started, higher lipid saturation and high oxygen concentrations will enhance the velocity of lipid radical chain reactions (Abele, & Puntarulo, 2004). In fact, in a previous study conducted by the authors (Anacleto *et al.*, 2014b), a tissue and cellular damage in lipids (lipid peroxidation) occurred particularly in native clams, revealing a peak at 28 °C, while invasive species only increased after 36 °C. This process is indicative of an enhancement of ROS action in an organism's lipids, being considered to be one of the most frequent cellular injury mechanisms (Lesser, 2006). Moreover, native species revealed higher levels of PUFA than invasive species, and this could partially explain the higher levels of lipid peroxidation observed between species. Interestingly, Anacleto *et al.* (2014b) observed that only native species had mechanisms of defence against thermal stress throughout a higher activity of antioxidant enzymes [glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD)] in order to remove ROS (produced during aerobic metabolism) and protect cells and their membranes and macromolecules from oxidation. Overall, glycogen and fatty acids decreased in warming environment in native clam, possibly as a consequence of higher energetic demand. Indeed, in this acute stress situation (involving the adjustment of an organism to an immediate change in temperature), the organisms usually use carbohydrates as a first energy source (either as free glucose or obtained by degradation of the glycogen stored in the digestive gland and mantle), followed by other energy sources such as fatty acids (Erk, Ivanković, D., & Stržak, 2011). High glycogen levels in bivalves may be interpreted as a metabolic adaptive strategy to endure environmental changes (Maazouzi *et al.*, 2011). In contrast, invasive species has a different adaptive strategy to cope with acute warming conditions, since this species has low glycogen, fatty acids and protein content and appears to use only fatty acids to endure environmental warming. Anacleto *et al.* (2014b) have previously observed that only this native clam species *R. decussatus* have physiological mechanisms of defence against

thermal stress, using energetically expensive cellular responses, contrarily to the same invasive clam species *R. philippinarum*. Indeed, this invasive species have not shown higher tolerance to heat stress, but adopted an inexpensive mechanism of defence against thermal stress, i.e. a behavioural strategy through the closure of valves during stressful short-term periods to ensure isolation from severe conditions and, consequently, guarantee longer survival.

In general, atherogenic (AI) and thrombogenic (TI) indices were low, especially in the invasive species (AI: 0.21–0.23; TI: 0.16–0.17) regardless of temperature, which is mostly due to the low saturated fatty acid content in this species (Table 1). These indices did not significantly vary with warming, except AI in the native species that significantly decreased after 22 °C (Table 1). The low AI and TI indices obtained in both clam species suggest that these food items can be used in a cardio-protective and antithrombogenic diet that is ascribed to the strong platelet-aggregation-inhibiting effect via prostanoid PGI3 production (Ulbricht, & Southgate, 1991). Concerning the ratio *n*-3/*n*-6 fatty acids, the native species revealed significantly higher values at 22 °C (5.52), while in the invasive species the highest values occurred at 28 °C (2.54; Table 1). The U.K. Department of Health sets an ideal *n*-3/*n*-6 ratio of 4:1 in the human diet and a minimum value of PUFA/SFA ratio recommended of 0.45 (HMSO, 1994). In all temperatures, clam species had n-3/n-6 ratios in the range of the recommended values, as well as had PUFA/SFA ratio well above 0.45, which emphasize the excellent nutritional value of clams, particularly the native one. Indeed, *n*-3 PUFA are fundamental to the prevention of human chronic inflammatory and cardiovascular diseases (Simopoulos, 1991), while a diet rich in *n*-6 PUFAs promote blood viscosity, vasospasm, vasoconstriction and shorter bleeding time (Simopoulos, 1999). In addition, the lower h/H index found in native species (1.78–2.29; Table 1) indicates that the regular consumption of these clam species induce a hypocholesterolemic effect.

4. Conclusion

The sessile nature and filter-feeding habits of clams renders them particularly susceptible to biochemical changes and vulnerable to climate change, such as ocean warming. The results obtained in this study clearly indicate that warming greatly affects the biochemical composition of two clam species (the native *R. decussatus* and the invasive *R. philippinarum*), particularly the fatty acid composition. Both clam species revealed a predominance of PUFA over SFA and MUFA even in a warming environment, as well as high *n*-3/*n*-6 and PUFA/SFA ratios, especially in the native clam, demonstrating that this species has better nutritional quality than the invasive one. However, the loss of some *n*-3 PUFA (in native species), as EPA (in both species) and DHA (in invasive species), can be considered as the most negative consequence of warming, since it contributes to the loss of prime quality fatty acids for human health. Nonetheless, the indices of fat quality, AI, TI and h/H remained at low levels even with warming water in both species, suggesting that these food items can be incorporated to a healthy cardio-protective diet. This study highlights the importance to undertake further research to understand and forecast the climate change effects on the nutritional quality of marine organisms.

Acknowledgments

The authors gratefully acknowledge to Sandro Pinto and Pedro Patacas for their support in clam collection. The first author acknowledges a PhD scholarship of the Portuguese Foundation for Science and Technology (FCT; Ref. SFRH/BD/69274/2010), whereas the last two authors were supported by a research contracts from the IF 2013 (RR) and 2008 (AM) Programs.

References

Abele, D., & Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology, 138,* 405– 415.

Anacleto, P., Barrento, S., Nunes, M. L., Rosa, R., & Marques, A. (2014a). Portuguese consumers' attitudes and perceptions of bivalve molluscs. *Food Control, 41,* 168–177.

Anacleto, P., Maulvault, A. L., Lopes, V. M., Repolho, T., Diniz, M., Nunes, M. L., *et al.* (2014b). Ecophysiology of native and alien invasive clams in an ocean warming context. *Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology, 175,* 28– 37.

Bandarra, N. M., Batista, I., & Nunes, M. L. (2009). Chemical composition and nutritional value of raw and cooked black scabbardfish (*Aphanopus carbo*). *Scientia Marina, 73S2,* 105-113.

Bandarra, N. M., Calhau, M. A., Oliveira, L., Ramos, M., Dias, M. G., Bartolo, H., *et al.* (2004). *Composição e valor nutricional dos produtos da pesca mais consumidos em Portugal.* Lisbon: IPIMAR, 103 p.

Barber, B. J., & Blake, N. J. (1981) Energy storage and utilisation in relation to gametogenesis in *Argopecten irradians concentricus* (Say). *Journal of Experimental Marine Biology and Ecology, 52,* 121–134.

Bayne, B. L., Thompson, R. J., & Widdows J. (1976). Physiology I. In B. L. Bayne (Ed.), *Marine mussels: their ecology and physiology* (pp. 121–206). Cambridge: Cambridge University Press.

Cardoso, C., Mendes, R., Vaz-Pires, P., & Nunes, M. L. (2009). Effect of dietary fibre and MTGase on the quality of mackerel surimi gels. *Journal of the Science of Food and Agriculture, 89,* 1648–1658.

Cohen, Z., Vonshak, A., & Richmond, A. (1988). Effect of environmental conditions on fatty acid composition of the red algae *Porphyridium cruentum*: correlation to growth rate. *Journal of Phycology, 24,* 328–332.

Dittrich, B. (1991). Biochemical composition of the parasitic amphipod *Hyperia galba* in relation to age and starvation. *Journal of Comparative Physiology B, Biochemical, Systemic, and Environmental Physiology, 161,* 441–449.

Dong, F. M. (2001). *The nutritional value of shellfish.* University of Washington: Washington Sea Grant Program. [Internet document] URL: http://www.wsg.washington.edu/communications/online/shellfishnutrition_09.pdfAccessed March, 26, 2014.

Erk, M., Ivanković, D., & Strižak, Z. (2011). Cellular energy allocation in mussels (*Mytilus galloprovincialis*) from the stratified estuary as a physiological biomarker. *Marine Pollution Bulletin, 62,* 1124–1129.

FAO (Food and Agriculture Organization of the United Nations) (2008). *Climate change: implications for food safety.* Rome, Italy: FAO. [Internet document] URL: http://www.fao.org/docrep/010/i0195e/i0195e00.HTM. Accessed April, 07, 2014.

FAO (Food and Agriculture Organization of the United Nations) (2014). *Fisheries and Aquaculture Information and Statistics Service.* [Internet document] URL: http://www.fao.org/fishery/statistics/en. Accessed April, 07, 2014.

Flassch, J. P., & Leborgne, Y. (1992). Introduction in Europe, from 1972 to 1980, of the Japanese Manila clam (*Tapes philippinarum*) and the effects on aquaculture production and natural settlement. *ICES Marine Science Symposia, 194,* 92–96.

Gabbott, P. A. (1983). Developmental and seasonal metabolic activities in marine molluscs. In: P.W. Hochachka (Ed.), *The mollusca, 2, environmental biochemistry and physiology* (pp. 165–217). New York: Academic Press.

Gonçalves, A., Pedro, S., Duarte, A., & Nunes, M. L. (2009). Effect of enriched oxygen atmosphere storage on the quality of live clams (*Ruditapes decussatus*). *International Journal of Food Science & Technology, 44,* 2598–2605.

HMSO (1994). *Nutritional aspects of cardiovascular disease: report on health and social subjects.* Committee of Medical Aspects of Food Policy, 46, Department of Health, London, UK: HMSO.

Hochachka, P. W., & Somero, G. N. (2002). Biochemical adaptation: mechanism and process in physiological evolution. Oxford: Oxford University Press, 560 p.

IPCC. (2013). Technical Summary. In: T. F., Stocker, Qin, D., Plattner, G. K., Tignor, M., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex,V., & Midgley, P. M. (Eds.), *Climate change 2013: the physical science basis. Contribution of working group I to the fifth assessment report of the Intergovernmental Panel on Climate Change (IPCC)*, Cambridge, UK and New York, USA: Cambridge University Press.

Kayama, M., Hirata, M., & Hisai, T. (1986). Effect of water temperature on the desaturation of fatty acids in carp. *Bulletin of the Japanese Society for the Science of Fish, 52,* 853–857.Lake, I. R., Hooper, L., Abdelhamid, A., Bentham, G., Boxall, A. B. A., Draper, A., *et al.* (2012). Climate change and food security: health impacts in developed countries. *Environmental Health Perspectives, 120(11),* 1520–1526.

Lenz, M., da Gama, B. A. P., Gerner, N. V., Gobin, J., Gröner, F., Harry, A., *et al.* (2011). Non-native marine invertebrates are more tolerant towards environmental stress than taxonomically related native species: Results from a globally replicated study. *Environmental Research, 111,* 943–952.

Lesser, M. P. (2006). Oxidative stress inmarine environments: biochemistry and physiological ecology. *Annual Review of Physiology, 68,* 253–278.

Leyva, A., Quintana, A., Sánchez, M., Rodríguez, E. N., Cremata, J., & Sánchez, J. C. (2008). Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: method development and validation. *Biologicals, 36,* 134–141.

Lockwood, B. L., & Somero, G. N. (2011). Invasive and native blue mussels (genus *Mytilus*) on the California coast: the role of physiology in a biological invasion. *Journal of Experimental Marine Biology and Ecology, 400,* 167–174.

Maazouzi, C., Piscart, C., Legier, F., & Hervant, F. (2011). Ecophysiological responses to temperature of the "killer shrimp" *Dikerogammarus villosus*: is the invader really stronger than the native *Gammarus pulex*? *Comparative Biochemistry and Physiology, Part A: Molecular & Integrative Physiology, 159,* 268–274.

Makoto, O., Masazumi, N., & Tadashi, N. (1989). Involvement of prostaglandins in the spawning of the scallop, *Patinopecten yessoensis*. *Comparative Biochemistry and Physiology Part C Comparative Pharmacology, 94,* 595–601.

Marques, A., Nunes, M. L., Moore, S. K., & Strom, M. S. (2010). Climate change and seafood safety: human health implications. *Food Research International, 43(7),* 1766–1779.

Matoo, O. B., Ivanina, A. V., Ullstad, C., Beniash, E., & Sokolova, I. M. (2013). Interactive effects of elevated temperature and CO_2 levels on metabolism and oxidative stress in two common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). *Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology, 164(4),* 545–553.

Matozzo, V., Chinellato, A., Munari, M., Finos, L., Bressan, M., & Marin, M. G. (2012). First evidence of immunomodulation in bivalves under seawater acidification and increased temperature. *Plos One, 7(3),* e33820.OECD–FAO (2013). *OECD–FAO Agricultural outlook 2013–2022*. Paris, France: Organisation for Economic Co-operation and Development (OECD) publishing and Food and Agriculture Organization (FAO) of the United Nations. [Internet document] URL: www.oecd.org/site/oecd-faoagriculturaloutlook/. Accessed April, 07, 2014.

Oliveira, M.C.R.M. (2012). *Moluscos bivalves em Portugal: composição química e metais contaminantes.* Lisboa: Tese de Mestrado em Tecnologia e Segurança Alimentar, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, 85 p.

Olyarnik, S. V., Bracken, M. E. S., Byrnes, J. E., Hughes, A. R., Hultgren, K. M., & Stachowicz, J. J. (2009). Ecological factors affecting community invasibility. In: G., Rilov, & J.A. Crooks (Eds.), *Biological invasions in marine ecosystems: ecological, management, and geographic perspectives* (pp. 215–240). Heidelberg, Germany: Springer.

Patrick, S., Faury, N., & Goulletquer, P. (2006). Seasonal changes in carbohydrate metabolism and its relationship with summer mortality of Pacific oyster *Crassostrea gigas* (Thunberg) in Marennes-Oleron bay (France). *Aquaculture, 252,* 328–338.

Pernet, F., Tremblay, R., Comeau, L., & Guderley, H. (2007). Temperature adaptation in two bivalve species from different thermal habitats: energetics and remodelling of membrane lipids. *Journal of Experimental Biology, 210,* 2999–3014.

Saint-Denis, T.,& Goupy, J. (2004). Optimization of a nitrogen analyser based on the Dumas method. *Analytica Chimica Acta, 515(1),* 191–198.

Santos-Silva, J., Bessa, R. J. B., & Santos-Silva, F. (2002). Effect of genotype, feeding and slaughter weight on the quality of light lambs II. Fatty acid composition of meat. *Livestock Production Science, 77,* 187–194.

Sargent, J., Bell, G., McEvoy, L., Tocher, D., & Estevez, A. (1999). Recent developments in the essential fatty acid nutrition of fish. *Aquaculture, 177,* 191–199.

Simopoulos, A. P. (1991). Omega-3 fatty acids in health and disease and in growth and development. *American Journal of Clinical Nutrition, 54,* 438–463.

Simopoulos, A. P. (1999). Essential fatty acids in health and chronic disease. *American Journal of Clinical Nutrition, 70,* 560S–569S.

Sorte, C. J. B., Williams, S. L., & Zerebecki, R. A. (2010). Ocean warming increases threat of invasive species in a marine fouling community. *Ecology, 91(8),* 2198–2204.

Soudant, P., Van Ryckeghem, K., Marty, Y., Moal, J., Samain, J. F., & Sorgeloos, P. (1999). Comparison of polar lipid class and fatty acid composition between a reproductive cycle in nature and a standard hatchery conditioning of the pacific oyster *Crassostrea.gigas*. *Comparative Biochemistry and Physiology B Comparative Biochemistry, 123,* 209–222.

Streftaris, N., & Zenetos, A. (2006). Alien marine species in the Mediterranean - the 100 'worst invasives' and their impact. *Mediterranean Marine Science, 7,* 87–118.

Tirado, M. C., Clarke, R., Jaykus, L. A., McQuatters-Gollop, A., & Frank, J. M. (2010). Climate change and food safety: a review. *Food Research International, 43,* 1745–1765.

Ulbricht, T. L., & Southgate, D. A. T. (1991). Coronary heart disease: seven dietary factors. *Lancet, 338,* 985–992.

Van Dooremalen, C., Koekkoek, J., & Ellers, J. (2011). Temperature-induced plasticity in membrane and storage lipid composition: thermal reaction norms across five different temperatures. *Journal of Insect Physiology, 57(2),* 285-291.

Viles, P., & Silverman J. (1949). Determination of starch and cellulose with anthrone. *Journal of Analytical Chemistry, 21,* 950–953.

Weihrauch, J. L. Posati, L. P., Anderson, B. A., & Exler, J. (1977). Lipid conversion factors for calculating fatty acids contents in foods. *Journal of the American Oil Chemists' Society, 54,* 36–40.

CHAPTER 9

9. General discussion and conclusions

9.1 Microbiological and chemical safety of native and invasive clams from Tagus estuary

9.1.1 Bivalve consumers' behaviour

9.1.2 Microbiological contamination of clams

9.1.3 Microbiological and chemical responses to depuration

9.1.4 Microbiological responses to transport

9.2 Physiological responses and nutritional quality of native and invasive clams

9.2.1 Responses to depuration and transport

9.2.2 Responses to environmental warming

9.2.3 Nutritional quality in environmental warming

9.3 Perspectives

9.4 References

9. GENERAL DISCUSSION AND CONCLUSIONS

The present thesis reveals the first comprehensive and comparative assessment of the microbiological, physiological and chemical responses of native and invasive clams to depuration, transport and environmental stress. Such research is particularly relevant and useful because: 1) bivalves have a high relevance in Portuguese economy and gastronomy; 2) bivalve consumption behaviour has specificities that may lead to higher health risks for consumers, such as the preference for live/raw or lightly cooked bivalves, and summer months for its consumption (see details in Chapter 2); 3) bivalve production areas are constantly vulnerable to microbiological and chemical contamination; 4) the trade chain of live bivalves is complex and includes several steps usually performed under sub-optimal conditions, affecting negatively the physiological condition of the animals (see details in Chapter 5); 5) environmental warming is expected to affect physiological and biochemical responses of bivalves, since they inhabit in inshore estuaries subjected to strong anthropogenic pressures, which will lead to alterations in their nutritional quality and safety; and 6) invasive species are expected to be more adapted to environmental variations than native species.

9.1 Microbiological and chemical safety of native and invasive clams from Tagus estuary

9.1.1 Bivalve consumers' behaviour

The national survey, presented in chapter 2, was the first approach to characterize the profile of bivalve's consumers, the most important quality criteria involved in bivalve purchase and predict the main risks associated with their consumption. The majority of Portuguese consumers of bivalves, consume clams such as pullet carpet shell clam *Venerupis pullastra*, grooved carpet shell clam *Ruditapes decussatus* and Japanese carpet shell clam *Ruditapes philippinarum* (26.8%, 20.1% and 19.5%, respectively; Fig. 1 in Chapter 2), being these species among the main harvested species in Tagus estuary (Chapter 3). The majority of consumers prefer to eat clams in summer (Table 1; Table 3 in Chapter 2). The most relevant quality criteria associated to the purchase of clams are the price, trust in the selling establishment and product's label information (expiry date, origin, size and smell; Table 5 in Chapter 2). On the other hand, several risks associated with clams' consumption were pointed out by consumers and perceived through several answers. Even though the majority of consumers are aware about the risks associated

with bivalve consumption, pointing out food poisoning, toxins and heavy metals as the main risks, still a significant percentage (25%) of them have no knowledge (Table 1; Table 3 in Chapter 2).

Table 1. Main risks associated with bivalve molluscs' consumption and higher-risk population.

Risks	Portuguese consumers (%)	Main consumers' categories
Purchase: collection or directly to the fisherman	18	▪ <25 years old (35%) ▪ males (26%) ▪ with basic education level (24%) ▪ ≥2 members in the household (18%) ▪ with household income level ≤800€ (26%) ▪ living in coastal region (19%)
Season of consumption: summer	54	▪ <25 years old (62%) ▪ with higher education level (55%) ▪ single (57%) ▪ with household income level ≤800€ (59%) ▪ living in coastal region (55%)
Mode of consumption: raw	2	▪ 55–65 years old (3%) ▪ males (3%) ▪ with basic education level (3%) ▪ single (4%) ▪ with household income level >3000€ (2%) ▪ living in interior region (3%)
Mode of consumption: slightly cooked	86	▪ 35–44 and >65 years old (92% and 91%, respectively) ▪ with higher education level (88%) ▪ single (90%) ▪ with household income level >3000€ (89%) ▪ living in coastal region (87%)
Consumption of broken or closed shells after cooking	19	▪ >65 years old (36%) ▪ males (24%) ▪ with basic education level (27%) ▪ ≥2 members in the household (20%) ▪ with household income level ≤800€ (25%) ▪ living in coastal region (19%)
Without knowledge of risks of clams' consumption	25	▪ <25 years old (37%) ▪ women (26%) ▪ with basic education level (38%) ▪ ≥2 members in the household (25%) ▪ with household income level ≤800€ (32%) ▪ living in coastal region (25%)

In fact, risky behaviours are mentioned by consumers, including the harvest of specimens from polluted areas or direct purchase of bivalves to fisherman (18%), often without information about the safety of the harvesting site, and some also consume raw or lightly steamed clams (88%), even with broken and/or closed shells after cooking (19%) (Table 1; Table 3 in Chapter 2). Such practices may pose a substantial risk to human health, since they represent a potential source of serious infections and diseases to consumers (Gerba, 1988), even when shortly cooking practices are used that rarely heats bivalve flesh to temperatures that effectively inactivate most pathogenic agents (Koff and Sear, 1967). Portuguese consumers' behaviour, i.e. attitudes and preferences, differ considerably

according to their demographic and socio-economic characteristics. Particularly young consumers (<25 years old), males, with basic education level, with lower household monthly income level (≤800€) and living in the coastal regions are among the main clams' consumers with higher-risk of having bivalve-derived diseases (Table 1). This indicates that there is a need to develop education strategies targeted to specific segments of the population to increase the awareness for risky practices.

9.1.2 Microbiological contamination of clams

There is a growing awareness about the problems associated with the introduction of biologic invasive species, because they are among the most serious global environmental threats to biodiversity, human health and food security (Sala *et al.*, 2000). From the microbiological point of view, *R. philippinarum*, an invasive species introduced in the last decade in Portuguese estuaries, revealed higher contamination level (in terms of *E. coli*, *Vibrio* spp., *Salmonella* spp. and TVC), reflecting the lower quality of the surrounding waters and sediment where the species live (Barreiro) in comparison with the native species, *V. pullastra* (Trafaria; Table 2; Chapter 3). This is of particular relevance since the consumption of this species has increased significantly in the last years among the Portuguese population (Chapter 2).

Table 2. Seasonal bacterial variation (*E. coli*, *Vibrio* spp., TVC and *Salmonella* spp.) in clams (*Venerupis pullastra* and *Ruditapes philippinarum*), water and sediment from Trafaria and Barreiro.

		Venerupis pullastra	***Ruditapes philippinarum***
E. coli	Contamination level	C ≈ S > SW	C > S > SW
	Highest level (log CFU/100 mL or log CFU/100 g)	**SW:** 2.7 (November) **C:** 3.3 (July) **S:** 2.7 (October)	**SW:** 3.5 (November) **C:** 5.3 (June) **S:** 4.1 (January)
***Vibrio* spp.**	Contamination level	S > C > SW	S > C > SW
	Highest level (log CFU/mL or log CFU/g)	**SW:** 0.7 (October–November) **C:** 2.5 (October) **S:** 3.2 (July)	**SW:** 2.1 (August) **C:** 2.9 (September) **S:** 4.2 (July, September)
TVC	Contamination level	S > C > SW	S > C > SW
	Highest level (log CFU/100 mL or log CFU/100 g)	**SW:** 3.0 (November) **C:** 4.1 (October) **S:** 5.3 (August)	**SW:** 3.5 (November) **C:** 5.1 (January, July) **S:** 5.6 (September)
***Salmonella* spp.**	Highest frequency	clam (December)	clam (January, April, May)

Abbreviations: SW, seawater; S, sediment; C, clams, CFU, colony-forming units, TVC, total viable counts.

A strong influence of seasonal and environmental parameters was detected on the occurrence of bacteria in both clam species, such as: 1) in summer, significantly higher *Vibrio* spp. levels were found (particularly in *R. philippinarum* and sediment samples),

when lower rainfall levels and higher salinity and temperature were measured; 2) in autumn-winter, significantly higher TVC and *E. coli* levels were observed (particularly in water and sediment samples), when higher dissolved oxygen, pH and rainfall, as well as lower temperature and salinity was observed; and 3) the presence of *Salmonella* spp. (particularly in both clams and *R. philippinarum* sediment) generally occurs when higher levels of indicators of faecal pollution (*E. coli*) were detected (Table 2; Figs. 2-4 and Table 3 in Chapter 3). Other favourable conditions to *E. coli* survival observed by Campos *et al.* (2013) are low solar radiation, low densities of micro-predators and high levels of organic matter. Overall, rainfall was the abiotic factor described by several authors (Setti *et al.*, 2009; Almeida and Soares, 2012; Bettencourt *et al.*, 2012; Campos *et al.*, 2013) that mostly influence the levels of microbiological contamination, particularly in *E. coli* levels and in the incidence of *Salmonella* spp. This environmental parameter leads to an increase in the transport of contaminants through runoff of waters from rural and urbanized areas that flow into estuaries increasing faecal contamination (Campos *et al.*, 2013). Indeed, several industries and farming facilities located nearby estuaries, combined with a highly populated area, contribute to the production of heavily polluted wastewaters, explaining the higher *E. coli* levels detected in *R. philippinarum* clams, water and sediment. Some studies have shown that *E. coli* and *Salmonella* spp. may adapt to the marine environment, remaining viable and culturable for long periods of time, if cells are in a suitable nutrient-rich environment, like organically polluted waters (Timoney and Abston, 1984; Martinez-Urtaza *et al.*, 2004).

Regarding *Vibrio* species, the environmental prevalence of these bacteria displays a strong seasonality, being temperature and salinity among the dominant physico-chemical drivers (Thompson *et al.*, 2003). Thus, the sensitivity of *Vibrio* species to cold temperatures are in agreement with the findings of others researchers (Ripabelli *et al.*, 1999; De Paola *et al.*, 2003), where a positive correlation between the water temperature (>15 °C) and the detection of *Vibrio* spp. was detected (Lhafi and Kühne, 2007). However, an adaptation of the pathogens *V. vulnificus* and *V. parahaemolyticus* to cooler water temperatures can also occur (between 8 and 14 °C) (Wright *et al.*, 1996; Høi *et al.*, 1998; Oliver, 2005), indicating that *Vibrio* spp. enters in a viable but non-culturable state below 7.6 °C. In this way, the survival and persistence of *Vibrio* species under unfavourable growth conditions involves a dormant state characterized by a decrease in metabolic rate, loss of the flagellum, conformational change to a small sphere and an inability to grow on standard microbiological media (Oliver, 2005; Turner, 2010). Another mechanism pointed out for *Vibrio* spp. survival involves the ability to associate with biotic and abiotic surfaces such as particulate organic matter (POM), plankton and sediment (Heidelberg *et al.*, 2002; Turner

et al., 2009). In fact, the highest levels of *Vibrio* spp. was found in sediment rather than in clams or water as well as a positive correlation between sediment and both clam species and *R. philippinarum* surrounding water (see Table 4 in Chapter 3).

9.1.3 Microbiological and chemical responses to depuration

According to the current classification of harvesting areas, both clam species harvested in Tagus estuary reveal *E. coli* levels well above the legal limit (230 CFU/100 g of wet meat; EC, 2004), with *V. pullastra* being from B category harvesting areas (Trafaria local) and *R. philippinarum* from B or C categories (Alcochete and Barreiro locals, respectively; see Fig. 2 in Chapter 3 and Fig. 4 in Chapter 4). Despite the fact that depuration is a mandatory treatment to eliminate *E. coli levels* in these bivalve species, it is often an uncommon practice among clam fisherman's and marketers, being specimens often sold without sanitary control to wholesalers, retailers or consumers. The results obtained in chapter 4 for both clams' species allowed to conclude that depuration is an important and efficient process in the reduction of *E. coli* and TVC levels, particularly after 24 and 48h, respectively, but not applicable for *Vibrio* spp. (Table 3; Figs. 3, 4 and 5 in Chapter 4), which might be due to the colonization of this bacterium in bivalves' intestinal tracts (Su and Liu, 2007; Martínez *et al.*, 2009).

Table 3. Microbiological responses (TVC, *E. coli* and *Vibrio* spp. levels) of native (*Venerupis pullastra*, VP) and invasive clams (*Ruditapes philippinarum*, RP) to depuration and semidry simulated transport at two temperatures (4 and 22 °C).

Bacteria	Species	Depuration	Depuration + Transport		Transport (without depuration)	
			4 °C	22 °C	4 °C	22 °C
TVC (CFU/g)	VP	↓ 0.6 log (48h)	< RL (until LT50)	> RL (after 0h)	< RL (until 72h)	< RL (until 24h)
	RP	↓ 0.8 log (48h)	< RL (until 96h)	< RL (until 24h)		
E. coli (CFU/100g)	VP	UL (< LL, 24h)	UL (< LL, after 0h)	UL (< LL, after 0h)	< LL (after 24h)	< LL (after 24h), UL at 72h
	RP				>LL	< LL (after 48h)
Vibrio spp. (CFU/g)	VP	↑ 1.0 log (48h)	constant	↑ 2.7 log (LT50)	↑ 0.4 log (LT50)	↑ 3.6 log (LT50)
	RP	↑ 0.6 log (48h)	↑ 0.5 log (LT50)	↑ 4.6 log (LT50)	↑ 1.1 log (LT50)	↑ 5.2 log (LT50)

Abbreviations: TVC, total viable counts; CFU, colony-forming units; RL, recommended limit (5.7 log CFU/g; ICMSF, 1986); UL, undetected limits; LL, legal limit (230 CFU/100 g of wet meat; EC, 2004).

Although bacteria reduction depends of initial levels of contamination and bivalve species, a high efficacy of the depuration in *E. coli* levels (>90%) after a relatively short period of time (24h) was found by several authors for other clam species, namely *Chamelea gallina* (Barile *et al.*, 2009), *Egeria radiate* (Ekanem and Adegoke, 1995) and mussel species, *Mytilus galloprovincialis* (Croci *et al.*, 2002). Despite TVC levels also decreased with depuration in *R. phyllippinarum* and *V. pullastra* harvested from Tagus estuary, the initial values found were always below the recommended limit (5.7 log CFU/g; Fig. 3 in Chapter 4) for good quality fresh and frozen bivalve molluscs from the International Commission on Microbiological Specifications for Foods (ICMSF, 1986) as well as the levels observed throughout the year (Fig. 3 in Chapter 3).

Concerning *Vibrio* spp., several authors indicate that depuration only can be effective to reduce the levels of this bacteria if longer periods (44h to 10 days) were used, depending on the temperature used during the process since this parameter greatly influences *Vibrio* growth (Croci *et al.*, 2002; Cozzi *et al.*, 2009; López-Joven *et al.*, 2011). In fact, each bivalve species pump water over a certain temperature range and water-pumping activity can be affected by seawater temperature (Richards, 1991; Roderick and Schneider, 1994). Thus, bivalves that normally grow in cold water tend to have a lower optimal depuration temperature than those grown in warmer water (López-Joven *et al.*, 2011). For example, Yang (2008) reported that low-temperature depuration is a simple, successful and economical measure to reduce the risk of *V. parahaemolyticus* infection associated with raw oyster (*Crassostrea gigas*) consumption and can easily be adopted by the industry to provide safe oysters for consumption. Therefore, other efficient treatments are envisaged to efficiently decrease *Vibrio* spp. levels in bivalves, because unfortunately the condition and bivalve quality will progressively be reduced with time due to the lack of feed, representing significant economic losses to stakeholders if periods above 48h are employed in depuration facilities. In addition, since *E. coli* is an insufficient microbiological indicator to evaluate the quality and healthiness of bivalves and the current European legislation does not specify limits for *Vibrio* spp. (EC, 2001), it is fundamental that preventive measures are implemented for this bacterium, taking into account that they are naturally found in seawater and normal constituents of mollusc flora (Barile *et al.*, 2009), and that some strains (mainly *V. parahaemolyticus*) are also major health concerns in bivalves (Wittman and Flick, 1995; Mead *et al.*, 1999).

Concerning chemical contaminants (mainly Cd, Pb, As and Hg), the initial levels widely varied between bivalve species (*R. philippinarum*, *M. galloprovincialis* and *Scrobicularia*

plana; Chapter 6), mainly due to the chemical pollution from the industries near the harvesting areas and to the biological characteristics of bivalve species (clams usually live burrowed in sediment, while mussels are intertidal organisms). Nonetheless, depuration can be also effective in reduction of levels of toxic elements (mainly Pb) in the three species, but particularly in peppery furrow shell clam, *S. plana* (Table 4; Fig. 3; Tables 3 and 4 in Chapter 6) after two and eight depuration days (39 and 60%, respectively). This species (harvested in Tagus estuary) is currently declared unfit for human consumption due to the high levels of Pb DR, 2013) often found above the Maximum Permissible Limits (MPLs; 1.5 mg/kg; EC, 2006). Therefore, this study indicates that depuration may be employed as an excellent mitigation strategy to reduce the levels of this toxic element in contaminated bivalves to acceptable values for commercialization and human consumption as far as toxic elements are concerned. In relation to the other toxic elements, the levels in all studied bivalves' species were always well below the MPLs for Hg and Cd (0.5 and 1.0 mg/kg, respectively; EC, 2006) and the maximum allowable levels for total As (86 mg/kg; FDA, 1993), despite the depuration reduced Hg (32%; after six days), Cd (38%; after eight days) and As (19%; after four days) levels in *R. philippinarum* as well as 10% of As (after four days) in *S. plana* (Table 4; Fig. 3; Tables 3 and 4 in Chapter 6). Moreover, in general, other elements were significantly reduced, namely Fe, Br and Rb after two depuration days, as well as Cu, Sr and As after four depuration days (Table 4; Fig. 3; Tables 3 and 4 in Chapter 6). The higher depuration rate of elements, such as Pb and Fe, possibly reflects their weak binding to bivalve tissues and, thus, can be rapidly eliminated by depuration process. Although the nutritional quality of bivalves can decrease during depuration, more future studies should target this important topic.

Overall, the effectiveness of the depuration depends on several factors: duration of the depuration, health status of bivalves, environmental parameters in the depuration facility (e.g. temperature, salinity), the type and levels of contaminant (Jackson and Ogburn, 1999). Additionally, bivalves have different filtration and excretion rates, as well as different affinities to uptake and metabolize the contaminants. Another explanation for the reduction of some elements is related to the existence of non-enzymatic proteins, designated by metallothioneins, that have high affinity for elements like Cd, Hg, Cu and Zn, acting as a protective mechanism against the contaminant toxicity by sequestering and detoxifying these elements, though this has not been investigated in this thesis. Indeed, bivalves are known to have a great ability to regulate the levels of essential elements concentration and the detoxification process of non-essential elements (Thorsen *et al.*, 2007). Several studies have demonstrated that certain non-essential elements may share binding sites with essential elements (Suter, 2007). For instance, Ca can inhibit the uptake

of Cd through Ca channel blockers (Roesijadi and Unger, 1993; Wang and Evans, 1993; Qiu *et al.*, 2005).

Table 4. Significant reduction/increase (%) on concentration of essencial and toxic elements of three bivalves' species depurated (*Ruditapes philippinarum,* RP, *Mytilus galloprovincialis,* MG and *Scrobicularia plana,* SP).

Species	Element reduction/increase	Depuration time (days)			
		2	4	6	8
RP	Cl	↑ **48**			
	Fe	↓ 68	↓ 64	↓ **70**	↓ 52
	Br		↓ 25	↓ **27**	↓ 22
	Cu		↓ 33	↓ **37**	↓ 29
	Se			↓ **24**	
	Rb	↓ 61	↓ **68**	↓ 64	↓ **68**
	Sr			↑ **32**	↑ 5
	Hg			↓ **32**	
	Cd				↓ **38**
	Pb	↓ **71**	↓ 53	↓ 69	↓ 68
	As		↓ **19**	↓ 19	
MG	S		↑ 17		↑ **25**
	Cl		↓ 11		↓ **13**
	Fe	↓ 63	↓ 68	↓ **73**	↓ 67
	Zn		↑ **48**	↑ 46	↑ 44
	Br	↓ 21	↓ **44**	↓ 42	↓ 39
	Sr		↓ **39**	↓ 32	↓ 13
	Pb	↓ **27**			
SP	Cl	↑ **53**	↑ 25	↑ 45	↑ 29
	Fe	↓ 57	↓ 56	↓ 39	↓ **66**
	Zn			↑ 45	↑ **64**
	Br	↑ 53	↑ 29	↑ 56	↑ **58**
	Cu		↓ **28**		
	Rb	↓ 66	↓ 66	↓ 66	↓ **67**
	Sr			↑ 41	↑ **44**
	Pb	↓ 39	↓ 45	↓ 24	↓ **60**
	As		↓ **10**		

Bold numbers indicate maximum reduction/increase.

9.1.4 Microbiological responses to transport

Transport is another critical step of the trade chain of live bivalves since promotes stressful conditions to the organisms. The recommended conditions to transport live bivalves should be performed under controlled low temperatures (4 °C) in order to maintain the quality and safety of these products, avoiding stress to specimens (Lee *et al.*, 2008). However, in some cases, clams are packed in net 20 kg bags and subjected to wide temperature variations, since specimens are not maintained in chilling conditions during transport to depuration facilities. Thus, temperature conditions were tested in controlled environments to simulate semi-dry transport at optimal and stressful temperatures (4 and 22 °C, respectively). The results presented in chapter 4 highlighted the higher microbiological quality of both clam species (*V. pullastra* and *R. philippinarum*) transported at optimal conditions: i) *E. coli* levels decrease at slow rates, ii) TVC levels

increase at slow rates; and iii) *Vibrio* spp. levels remained constant. In contrast, clams transported at stressful conditions generally showed: i) a decrease in *E. coli* levels (always at fast growth rates); and ii) an increase in *Vibrio* spp. and in TVC levels (Table 3). On the other hand, it is important that clams are depurated beforehand to guarantee the safety of bivalves, as in optimal conditions (4 °C) they revealed: i) lower levels of TVC always below the recommended limit (until 120h and 96h for *V. pullastra* and *R. philippinarum*, respectively), ii) lower levels of *E. coli* within the safety limits for human consumption; and iii) constant *Vibrio* spp. levels. The inhibition of *Vibrio* species at chilling temperatures has also been highlighted in previous studies as well as their rapidly multiplication when exposed to elevated temperatures, e.g. *V. parahaemolyticus* in oysters maintained at 26 °C could increase rapidly to 50–790 folds within 24 h (Gooch *et al.*, 2002). Therefore, the only recommended way to protect consumers against contamination with pathogenic *Vibrio* strains is to cook clams before eating and to transport specimens in chilling conditions.

9.2 Physiological responses and nutritional quality of native and invasive clams

Physiological responses of bivalves can be used as effective parameters to identify stressful conditions (e.g. handling along the trade chain, environmental changes). Temperature is considered as one of the most important parameters affecting survival, physico-chemical and enzymatic reactions, physiological and biochemical processes, as well as ecological interactions, especially in ectothermic organisms like bivalves (Dunson and Travis, 1991; Hutchison and Dupré, 1992; Hochochka and Somero, 2002).

9.2.1 Responses to depuration and transport

In general, depuration did not negatively physiologically affect the studied bivalve species (Chapters 5 and 6), except *S. plana* that showed a greater utilization of biochemical reserves (i.e. glycogen content), resulting in high mortality levels, particularly after four depuration days (25% mortality rate and a decrease of 1.3 g/100g in glycogen; Table 1 and Figure 2 in Chapter 6). In fact, the reduction or depletion of glycogen reserves is often associated with mass mortalities of bivalve species (Uzaki *et al.*, 2003; Patrick *et al.*, 2006), since glycogen is the first energy reserve usually used for maintenance needs and to ensure bivalve capacity to sustain further stress (Patrick *et al.*, 2006). In contrast, depuration enables two additional days of survival of both clam species (*V. pullastra* and *R.*

philippinarum) until reaching LT50 during semi-dry storage at different temperatures (Table 5; Fig. 3 in Chapter 5).

Table 5. Physiological responses (mortality, condition index, breakdown products of ATP and glycogen content) of native (*Venerupis pullastra*, VP) and invasive clams (*Ruditapes philippinarum*, RP) to depuration and semidry simulated transport at two temperatures (4 and 22 °C).

Parameter	Species	Depuration (48h)	Depuration + Transport		Transport (without depuration)	
			4 °C	22 °C	4 °C	22 °C
Mortality (LT50)	VP	low (<3%)	2 + 5 days	2 + 3 days	2 + 5 days	2 + 3 days
	RP	low (<2%)	2 + 14 days	2 + 4 days	2 + 14 days	2 + 4 days
CI	VP	not varied	↓ after 1 day (slower until LT50)	↓ after 1 day (faster until LT50)	↓ after 3 days (slower until LT50)	↓ after 1 day (slower until LT50)
	RP				↓ after 1 day (faster until LT50)	
Nucleotides	VP	↑ ATP, ADP	↑ HxR, Hx ↓ ATP, ADP	↑ IMP, HxR, Hx ↓ ATP, ADP, AMP	↑ AMP, IMP, HxR, Hx	↑ ATP, HxR, Hx ↓ ADP, AMP
	RP	↓ ATP, HxR ↑ ADP	↑ ATP, HxR, Hx ↓ ADP, AMP		↑ ATP, ADP, IMP, Hx ↓ AMP	↑ IMP, HxR, Hx ↓ AMP
K-value (%)	VP	↓ 1.3	↑ 23% (until LT50)	↑ 21% (until LT50)	↑ 12% (until LT50)	↑ 27% (until LT50)
	RP	↓ 4.7	↑ 24% (until LT50)	↑ 36% (until LT50)	↑ 4% (until LT50)	↑ 21% (until LT50)
AEC value (%)	VP	↑ (<50%)	↓ after 1 day (<50%)	>50% (only at day 1)	<50%	
	RP		↓ after 3 days (<50%)	↓ after 1 day (<50%)		
Glycogen (g/100g)	VP	↓ 0.5	↓ 0.1(LT50)	↓ 0.4(LT50)	↓ 0.5(LT50)	↓ 0.8(LT50)
	RP	↓ 0.1	depleted (at LT50)			

Abbreviations: LT50, temperature required for 50% mortality; CI, condition index; AEC, adenylic energetic charge; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IMP, inosine monophosphate; HxR, inosine; Hx, hypoxanthine.

Among the several physiological parameters studied, mortality, condition index (CI), nucleotides (K-value) and glycogen content were considered as useful stress indicators for the physiological status of both bivalve species along the trade chain (see also Uzaki *et al.*, 2003; Pampanin *et al.*, 2005). The results presented in chapter 5 evidenced the better physiological conditions of both clam species transported at low temperature (4 °C), namely: i) higher survival rates (5 and 14 days for native and invasive clams, respectively); ii) slower decrease of CI; iii) slower ATP catabolism (HxR and Hx accumulation); iv) lower percentage of K-value and adenylate energy charge (AEC value); and v) slower decrease of glycogen content (Table 5). Indeed, CI reduction is accompanied by the reduction of glycogen reserves due to the stressful conditions during semi-dry storage (e.g. lack of feed), and if organisms are subjected to prolonged semi-dry storage,

shell gaping occurs followed by animal death (Laing and Spencer, 2006). Simultaneously, ATP catabolism and its breakdown products occur, i.e. the accumulation of HxR and Hx, which is connected with the enzymatic activity and bacterial growth (Surette *et al.*, 1988). In fact, higher bacterial levels are observed during semi-dry storage, particularly TVC and *Vibrio* spp. levels at 22 °C (Chapter 4). Throughout the experiments, the native clam revealed better physiological conditions (higher CI and glycogen content, but lower nucleotide K-value and AEC value), whereas the invasive clam showed higher survival rates, especially at 4 °C (e.g. *R. philippinarum* survived nine days more at 4 °C, but only one day more at 22 °C than *V. pullastra*; Table 5).

9.2.2 Responses to environmental warming

Invasive species tend to tolerate a wider range of temperatures and to display higher thermal tolerance than native species; consequently they are more able to physiologically respond to extreme conditions (Calosi *et al.*, 2008; Somero, 2010; Sorte *et al.*, 2010; Lockwood and Somero, 2011). However, the results obtained in chapter 7 showed that this is not the case for the species selected here, as native and invasive clams had similar thermal tolerance limits – i.e. identical LT50 (42 °C) and CTMax values (43 °C; Table 6; Figs. 1 and 2 in Chapter 7). It is also worth nothing that a higher vulnerability of native clams was observed when mortality starts to occur at 36 °C and 100% of mortality (LT100) occurs at 44 °C, while in the invasive species mortality occurs only after 40 °C and LT100 at 46 °C (Table 6). Several characteristics justify the invasive success of species, namely locals with broader temperature ranges and higher maximum temperatures, larger distributional range, better tolerance to environmental parameters, greater capacity to adapt to new environments and higher growth rate (Rejmánek, 1996, 2000). Yet, here one of the possible explanations for the invasion success of *R. philippinarum* is related with its life-history strategies, since this species has a more extended breeding period and a greater number of spawning events than *R. decussatus* (Laruelle *et al.*, 1994). These reproductive traits may confer an adaptive advantage.

Beyond the spread of invasive species, climate change is also among the most serious global environmental threats that may impact bivalves. Results presented in chapter 7 evidenced that warming had a strong effect on the metabolism of both clam species. Particularly, at lower temperatures (between 22–30 °C), the metabolic demand for oxygen increased with temperature with Q_{10} varying around 1.5 and 3.0 (Table 6; Figs. 3 and 4 in Chapter 7), indicating that both species have an active metabolic regulation and are not under thermal stress (Rosa and Seibel, 2008, 2010. These results can be explained by the

"oxygen and capacity-limitation of thermal tolerance" concept that links thermal tolerance windows directly to oxygen supply and energy demand (Pörtner *et al.*, 2004; Pörtner and Knust, 2007). Therefore, the changes in aerobic scope of bivalves with environmental warming are assumed not to be caused by lower levels of ambient oxygen, but rather by limited capacity of oxygen supply mechanisms (ventilatory and circulatory systems) to meet the animal oxygen demand (Pörtner and Knust, 2007).

Table 6. Physiological (CTMax, RMRs, Q_{10} values, HSP70/HSC70 levels, MDA and antioxidant enzyme activities) and biochemical responses (protein, glycogen and fatty acids contents) of native (*Ruditapes decussatus*, RD) and invasive clams (*Ruditapes philippinarum*, RP) to environmental warming.

Parameter	Native species (RD)	Invasive species (RP)
LT50	42 °C	42 °C
LT100	44 °C	46 °C
CTMax	42.6 ± 1.7 °C	43.1 ± 1.3 °C
RMRs (μmol O_2/g/h)	↑ 4.1 (22–38 °C) ↓ 4.6 (until LT50)	↑ 4.3 (22–30 °C) ↓ 6.8 (30–38 °C) ↑ 1.3 (until LT50)
P_{crit}	Oxyconformer	Oxyconformer
Q_{10} value	1.5 – 3.0 (22–30 °C) <1.5 (until LT50)	1.5 – 3.0 (22–30 °C) <1.5 (30–38 °C) 3.7– 8.5 (until LT50)
Valve closure behaviour	Valve gaping (except 20% between 32 and 35 °C)	Valves tightly closed (except ≥41 °C)
HSP70/HSC70 levels (μg/μg total protein)	↑ 0.030 (22–32 °C) ↓ 0.017 (until LT50)	≈ stable
MDA (nmol/min/μg total protein)	↑ 0.0017 (22–28 °C) ↓ 0.0027 (until LT50)	≈ stable (22–36 °C) ↑ 0.0006 (until LT50)
GST (nmol/min/μg total protein)	↑ 0.033 (22–30 °C) ↓ 0.0032 (until LT50)	≈ stable
CAT(nmol/min/μg total protein)	great oscillations (peak at 24 °C: 0.097; minimum at 42°C: 0.044)	≈ stable
SOD (U/μg total protein)	↑ 0.017 (22–30 °C) ↓ 0.023 (until LT50)	≈ stable
Protein	≈ stable	≈ stable (peak at 34 °C)
Glycogen	great oscillations (minimum at 32 and 34 °C)	≈ stable
Fatty acids	↓ SFA, MUFA, PUFA, *n*-3/*n*-6 ratio, total FA, AI	↓ SFA, MUFA, PUFA, total FA

Abbreviations: LT50, temperature required for 50% mortality; LT100, temperature required for 100% of mortality; CTMax, critical thermal maximum; RMRs, routine metabolic rates; P_{crit}, critical oxygen partial pressure; Q_{10}, thermal sensitivity; HSP70, heat shock protein 70; HSC70, heat shock cognate 70; MDA, malondialdehyde; GST, glutathione S-transferase; CAT, catalase; SOD, superoxide dismutase; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; FA, fatty acids; AI, atherogenicity index.

Moreover, both clam species are considered oxyconformer organisms, i.e. they regulate their O_2 consumption rate in a nearly linear function to the minimum O_2 levels encountered in their environments (Grieshaber *et al.*, 1994; Childress and Seibel, 1998; Boutilier, 2001). This means that their critical oxygen partial pressure (P_{crit}) values are

generally lower than the minimum O_2 level they encounter to favour diffusion pathways and O_2 uptake. On the other hand, at temperatures above 30 °C, both clams are under active metabolic depression, and the metabolism became independent of the temperature, as indicated by Q_{10} values below 1.5, reaching almost 0 (Table 6; Figs. 3 and 4 in Chapter 7).

It is also worth noting that the invasive species had extremely high Q_{10} values at higher temperatures (between 38 and 42 °C; Table 6; Fig. 4 in Chapter 7), which seems to be related to sudden behaviour change, i.e. the clams opened their valves under such harsh thermal conditions, after tightly close the valves at temperatures under 38 °C (Table 6; Fig. 5 in Chapter 7). This valve closure of invasive clams seems to be an adaptive behavioural strategy that can be efficient during stressful short-term periods to ensure isolation and guarantee longer survival, but will not be advantageous under the chronically warming conditions predicted in the future (up to 4.8 °C; IPCC, 2013), because it restricts gas exchange and, consequently, triggers metabolic arrest and the enhancement of anaerobic pathways.

Environmental conditions such as thermal stress leads to the production of reactive oxygen species (ROS), generated by successive transfer of electrons in the mitochondrial respiratory chain, which promotes an oxidative stress characterized by the enhancement of antioxidant defences (Lesser, 2006; Truebano *et al.*, 2010). Chapter 7 suggests that environmental warming only had a stronger effect in the oxidative status of the native clam *R. decussatus*, as indicated by the higher HSP70/HSC70 and MDA levels, as well as antioxidant enzyme activities (GST, CAT and SOD levels), with the higher levels being found around 30 °C (Figs. 3, 7 and 9 in Chapter 7). This finding contradicts most of the studies comparing invasive and native species that revealed higher tolerance to heat stress of the invasive species (Hofmann and Somero, 1996; Lockwood and Somero, 2011; Zerebecki and Sorte, 2011). However, Maazouzi *et al.* (2011) concluded that an invasive crustacean species (*Dikerogammarus villosus*) is more adapted to lower temperatures (5–10 °C) with a limited possibility of adjustment above 20 °C, whereby the global warming is likely to be less favourable to this species than the native one (*Gammarus pulex*). The adaptive strategy of this crustacean species will be demonstrated by reduced metabolic and activity rates, associated with higher glycogen content, which allows them to successfully invade harsh and/or unpredictable biotopes, being the glycogen stores possibly used as a powerful indicator of their optimal thermal window. Overall, the results presented in chapter 7 suggest that the native species use energetically expensive cellular responses to deal with thermal stress, contrarily to the invasive species. Other studies also

referred that stress responses differ according to specimens gender (Øverli *et al.*, 2006), age/size (Hall *et al.*, 2000) and habitat (Timofeyev *et al.*, 2009).

Native species showed the T_{on} (onset temperature) at 24 °C, the T_{peak} (temperature of maximal induction) at 28 °C and the T_{off} (cessation temperature of heat shock response, HSR) at 34 °C (Fig. 7 in Chapter 7). Thus, at temperatures above T_{peak}, HSP synthesis cannot match the increasing cellular thermal exposure, suggesting that $T_{off's}$ relevance is limited under natural conditions (Tomanek and Somero, 1999). Since HSR is a highly ATP-dependent mechanism, warming conditions may become energetically limiting for specimens, leading to a transition to an anaerobic mode of the mitochondrial metabolism and to the progressive insufficient energy at the cellular level (Pörtner, 2002, 2010). Thus, at LT50 there may not be sufficient cell ATP to enable HSP70 response. On the other hand, above the T_{off}, an extreme hyperthermia seems to reduce antioxidant enzymatic defences (Kregel, 2002; Abele and Puntarulo, 2004), explaining the reduction of GST, CAT and SOD activities, as well as the lower MDA concentrations (Table 6; Figs. 7B and 8 in Chapter 7). The decrease of CAT activity indicates a reduced activity to protect cells from the accumulation of H_2O_2, while the decrease in SOD activity is likely due to the reduced production of this enzyme's substrate (O_2^-) as a consequence of lower oxygen availability at higher temperatures.

9.2.3 Nutritional quality in environmental warming

From the biochemical point of view, native clams also revealed, during warming, significantly higher concentration of protein, glycogen and fatty acids than the invasive species, indicating that the native species has a better nutritional quality (Fig. 1 and Table 1 in Chapter 8). In addition, this finding is supported by *n*-3/*n*-6 and PUFA/SFA ratios that are in the range of the recommended values set by U.K. Department of Health (HMSO, 1994). Thus, chapter 8 reveals that environmental warming can affect the nutritional quality of both clam species, since glycogen (only in native species) and fatty acids contents (in both species) seem be used as energy source against thermal stress (Table 6; Fig. 1 and Table 1 in Chapter 8). Thus, this can be a consequence of the higher energetic demand. Indeed, in this acute stress situation (involving the adjustment of an organism to an immediate change in temperature), the organisms usually use carbohydrates as a first energy source (either as free glucose or obtained by degradation of the glycogen stored in the digestive gland and mantle), followed by other energy sources such as fatty acids (Erk *et al.*, 2011). Therefore, the use of the protein fraction as an energy source for the

maintenance of bivalve metabolic needs only takes place when carbohydrate and lipid reserves have already been greatly depleted (Barber and Blake, 1981).

Particularly the fatty acid composition significantly decreased with warming (generally above 22 °C), but the loss of *n*-3 PUFA (in the native species), EPA (in both species) and DHA (in the invasive species) was the most negative outcome derived from warming, since it contributes to the loss of prime quality fatty acids for human health (Table 1 in Chapter 8). This is particularly relevant as these fatty acids are essential for marine organisms in general (Sargent *et al.*, 1999) and for the human diet to enable the prevention of chronic inflammatory and cardiovascular diseases (Simopoulos, 1991). On the other hand, nutritional quality indices (AI, TI and h/H) remained low in both species, even in warming conditions, suggesting that these food items can be incorporated in a healthy cardio-protective and hypocholesterolemic diet (Table 1 in Chapter 8). It is worth nothing that some relevant fatty acids found in both clam species (20:1 and 22:1) did not decrease with warming, particularly in native species, since are generally from exogenous origin (e.g. available feed; Bandarra *et al.*, 2009).

Many aquatic organisms are adapted to temperature variations, allowing physiological processes and biochemical reactions to proceed efficiently (Brett and Groves, 1979). Thus, these organisms adjust the composition of their membrane lipids to minimize the effects of temperature variation (Farkas *et al.*, 1980). Some variation generally occur in the proportion of unsaturated fatty acids (Hilditch and Williams, 1964), which seems to be essential for the normal function of basal metabolic processes at different environmental temperatures (Chapman, 1969). The results presented in chapter 8 revealed that the highest values of fatty acids were found at lower temperatures (22 °C) and during the warming conditions a prevalence of PUFA over SFA and MUFA was detected in both clam species (Table 1 in Chapter 8). This finding is in accordance with others authors findings (Bell *et al.*, 1986; Henderson and Tocher, 1987; Williams and Somero, 1996; Hochachka and Somero, 2002; Pernet *et al.*, 2007), which referred that at lower temperatures (0 °C in bivalves) generally the PUFA content is higher, since these fatty acids are essential to maintain the fluidity and permeability of cell membranes in ectotherms. This thermal adaptation in biological membranes is designated by “homeoviscous adaptation (HVA)” (see Sinenky, 1974), and explains the patterns of temperature-induced change in membrane lipid structure and composition (Cossins and Sinensky, 1986; Hazel and Landrey, 1988). Therefore, as temperature is raised acutely, fluidity is increased beyond the optimal range and the membrane becomes "hyperfluid", while when temperature drops, membrane fluidity falls below the optimal range and membrane activities are

constrained, i.e. fatty acids become more unsaturated at low and more saturated at high temperatures (Hazel and Williams, 1990; Hazel, 1995; Hochachka and Somero, 2002; Van Dooremalen and Ellers, 2010). However, it was observed a decrease in the main SFA (PA and SA) with increasing temperature (Table 1 in Chapter 8), which is against the expected response because of its solidifying effects on membrane fluidity. Similar results were found by Van Dooremalen *et al.* (2011) in insect species, but other authors reported an increase in SFA with temperature increase in fish species (Skalli *et al.*, 2006; Akhtar *et al.*, 2014). Therefore, further studies should be performed using more gradual temperature changes, or organisms should be exposed to constant/fluctuations regimes of cold shock or heat shock.

9.3 Perspectives

Future studies still need to be undertaken in several bivalve species to assess the efficacy of the depuration process in reducing pathogenic *Vibrio* strains, such as *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* naturally accumulated in clams, as these strains are not eliminated with the current depuration conditions. Usually two variables should be considered and studied to improve depuration of Vibrios, namely seawater temperature and depuration time. Therefore, it is certainly needed to establish an optimal temperature of depuration according to the bivalve species, to avoid drastic conditions that could imply a stress for bivalves. Overall, it is fundamental that the authorities adapt the legislation in order to incorporate new microbial strains such as *Vibrio* spp. and not restrict only to *E. coli* in the monitoring programmes.

Other important and innovative tools, such as the high pressure processing (HPP) have to be further developed in bivalve species, because will be very useful for bivalves from highly contaminated areas (above category C), where they cannot be harvested for direct human consumption. This tool has been currently employed to enhance food safety, thereby decreasing the load of pathogenic foodborne bacteria. Some studies performed with *V. parahaemolyticus* and *V. vulnificus* in oysters have been successful, without degrading their nutritional quality and extending the shelf-life of this products (Cruz-Romero *et al.*, 2008; Kural and Chen, 2008; Kural *et al.*, 2008; Prapaiwong *et al.*, 2009). Since HPP has a threshold value specific to the inactivation of each microorganism, the best combination of pressure, temperature and holding time will have to be achieved to inactivate each pathogenic bacterium studied. Additionally, HPP is an effective tool to reduce the risk of infection, often caused by pathogenic *Vibrio* strains that are not eliminated by the depuration treatment. However, the negative aspects of HPP treatment

are the need of specific and high cost equipment and that bivalves are killed during processing.

As the accumulation of bacteria in bivalves is influenced by environmental and physico-chemical parameters, namely temperature, salinity, dissolved oxygen and pH (see Chapter 3), additional research is still needed to assess the effect of other environmental parameters in clams from Tagus estuary, such as precipitation, humidity, solar radiation, wind and tide range, in order to predict contamination levels in harvesting bivalve waters. In addition, it is expected that the occurrence of bivalves contaminated with pathogenic bacteria will increase in the future due to the effect of climate change. Since this is one of the greatest environmental threats that the world faces today and is expected to worsen during the next decades, it is of paramount importance to investigate its effect on the retention and bioaccumulation of pathogenic bacteria as well as other contaminants (e.g., viruses, HABs, toxic elements and organic pollutants (PCBs and PAHs)) by bivalves. Temperature, pH and dissolved oxygen (O_2) are the most important parameters that affect marine organisms, and that are expected to considerably change in the future. It is also important to evaluate the physiological, biochemical and behavioural responses of bivalves to climate change. Some findings have been described that current and future increases in these parameters are likely to have negative consequences for coastal bivalve populations and other marine calcifying organisms, such as in survival, growth, energy status, shell mechanical properties, ammonium excretion and absorption efficiency (Talmage and Gobler, 2011; Dickinson *et al.*, 2012; Fernández-Reiriz *et al.*, 2012). However, the interactions of these environmental parameters and also other stressors are still not entirely understood and need further research.

Hereafter, an assessment of the microbiological risks associated with bivalve consumption still needs to be developed to ensure that consumers are on the safe side. Quantitative microbial risk assessment (QMRA) is a new powerful statistical tool to evaluate the risk and severity of microbial foodborne diseases and to ensure the scientific basis for food safety control (Miliotis, 2007).

9.4 References

Abele, D., Puntarulo, S. 2004. Formation of reactive species and induction of antioxidante defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology,* 138(4), 405–415.

Akhtar, M.S., Pal, A.K., Sahu, N.P., Ciji, A., Mahanta, P.C. 2014. Higher acclimation temperature modulates the composition of muscle fatty acid of *Tor putitora* juveniles. *Weather and Climate Extremes,* 4, 19–21.

Almeida, C., Soares, F. 2012. Microbiological monitoring of bivalves from the Ria Formosa Lagoon (south coast of Portugal): a 20 years of sanitary survey. *Marine Pollution Bulletin,* 64, 252–262.

Bandarra, N.M., Batista, I., Nunes, M.L. 2009. Chemical composition and nutritional value of raw and cooked black scabbardfish (*Aphanopus carbo*). *Scientia Marina,* 73S2, 105-113.

Barber, B.J., Blake, N.J. 1981. Energy storage and utilisation in relation to gametogenesis in *Argopecten irradians concentricus* (Say). *Journal of Experimental Marine Biology and Ecology,* 52, 121–134.

Barile, N.B., Scopa, M., Nerone, E., Mascilongo, G., Recchi, S., Cappabianca, S., Antonetti, L. 2009. Study of the efficacy of closed cycle depuration system on bivalve molluscs. *Veterinaria Italiana,* 45(4), 555–566.

Bell, M.V., Henderson, R.J., Sargent, J.R. 1986. The role of polyunsatured fatty acids in fishes. *Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology,* 83, 711–719.

Bettencourt, F., Matias, D., Soares, F. 2012. Microbiological monitoring of bivalve molluscs growing areas: a food safety and public health approach. *Aquaculture Europe,* 37(3), 11–15.

Boutilier, R.G. 2001. Mechanisms of cell survival in hypoxia and hypothermia. *Journal of Experimental Biology,* 204, 3171–3181.

Brett, J.R., Groves, T.D.D. 1979. Physiological energetic. *In:* Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), *Fish Physiology: Bioenergetics and Growth*. Academic Press, New York, NY, pp. 279–351.

Calosi, P., Bilton, D.T., Spicer, J.I. 2008. Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biology Letters,* 4, 99–102.

Campos, C.J.A., Kershaw, S.R., Lee, R.J. 2013. Environmental influences on faecal indicator organisms in coastal waters and their accumulation in bivalve shellfish. *Estuaries and Coasts,* 36, 834–853.

Chapman, D. 1969. Physical studies of lipid–lipid and lipid–protein interactions. *Lipids,* 4(4), 251–260.

Childress, J.J., Seibel, B.A. 1998. Life at stable low oxygen levels: adaptations of animals to oceanic oxygen minimum layers. *Journal of Experimental Biology,* 201, 1223–1232.

Cossins, A.R., Sinensky, M. 1986. Adaptation of membranes to temperature, pressure, and exogenous lipids. *In:* Shinitzky, M. (Ed.), *Physiology of Membrane Fluidity*. CRC Press, Boca Raton, Fla, pp. 1-20.

Cozzi, L., Suffredini, E., Ciccaglioni, G., Croci, L., 2009. Depuration treatment of mussels experimentally contaminated with *V. parahaemolyticus* and *V. vulnificus*. *In: Proceeding ICMSS09*. Nantes, France, 6 pp.

Croci, L., Suffredini, E., Cozzi, L., Toti, L. 2002. Effects of depuration of molluscs experimentally contaminated with *Escherichia coli*, *Vibrio cholerae* O1 and *Vibrio parahaemolyticus*. *Journal of Applied Microbiology,* 92(3), 460–465.

Cruz-Romero, M., Kelly, A.L., Kerry, J.P. 2008. Effects of high-pressure on the microflora of oysters (*Crassostrea gigas*) during chilled storage. *Innovative Food Science and Emerging Technologies,* 9, 441–447.

De Paola, A., Nordstrom, J.L., Bowers, J.C., Wells, J.G., Cook, D.W. 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Applied and Environmental Microbiology,* 69 (3), 1521–1526.

Dickinson, G.H., Ivanina, A.V., Matoo, O.B., Pörtner, H.O., Lannig, G., Bock, C., Beniash, E., Sokolova, I.M. 2012. Interactive effects of salinity and elevated CO_2 levels on juvenile eastern oysters, *Crassostrea virginica*. *Journal of Experimental Biology,* 215, 29–43.

DR (Diário da República). 2013. *Despacho n.º 15264/2013 do Diário da República, 2.ª série, N.º 227, de 22 de Novembro de 2013.* pp. 34255–34258.

Dunson, W.A., Travis, J. 1991. The role of abiotic factors in community organization. *American Naturalist,* 138(5), 1067–1091.

EC (European Comission). 2001. Opinion of the Scientific Committee on veterinary measures relating to public health on *Vibrio vulnificus* and *Vibrio parahaemolyticus* (in raw and undercooked seafood). European Commission health & consumer protection directorate general. *Directorate C–Scientific Opinions,* 1–64.

EC (European Comission). 2004. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal of the European Union,* L226, 83–127.

EC (European Comission). 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006. Setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union,* L364, 5–24.

Ekanem, E.O., Adegoke, G.O. 1995. Bacteriological study of West African clam (*Egeria radiate* Lamarch) and their overlying waters. *Food Microbiology,* 12, 381–385.

Erk, M., Ivanković, D., Strižak, Z. 2011. Cellular energy allocation in mussels (*Mytilus galloprovincialis*) from the stratified estuary as a physiological biomarker. *Marine Pollution Bulletin,* 62, 1124–1129.

Farkas, T., Csengeri, I., Majoros, F., Olah, J. 1980. Metabolism of fatty acids in fish: III. Combined effect of environmental temperature and diet on formation and deposition of fatty acids in the carp, *Cyprinus carpio* Linnaeus 1758. *Aquaculture,* 20(1), 29–40.

FDA (Food and Drug Administration). 1993. *Guidance document for arsenic in shellfish.* Food and Drug Administration, U.S. Department of Health and Human Services, Public Health Service, Office of Seafood, Washington, DC, 44 pp.

Fernández-Reiriz, M.J., Range, P., Álvarez-Salgado, X.A., Espinosa, J., Labarta, U. 2012. Tolerance of juvenile *Mytilus galloprovincialis* to experimental seawater acidification. *Marine Ecology Progress Series,* 454, 65–74.

Gerba, C. 1988. Viral disease transmission by sea-foods. *Food Technology,* 42(3), 99–103.

Gooch, J.A., DePaola, A., Bowers, J., Marshall, D.L. 2002. Growth and survival of *Vibrio parahaemolyticus* in postharvest American oysters. *Journal of Food Protection,* 65, 970–974.

Grieshaber, M.K., Hardewig, I., Kreutzer, U., Pörtner, H.O. 1994. Physiological and metabolic responses to hypoxia in invertebrates. *Reviews of Physiology, Biochemistry and Pharmacology,* 125, 43–147.

Hall, D.M., Xu, L., Drake, V.J., Oberley, L.W., Oberley, T.D., Moseley, P.L., Kregel, K.C. 2000. Aging reduces adaptive capacity and stress protein expression in the liver after heat stress. *Journal of Applied Physiology,* 89, 749–759.

Hazel, J.R. 1995. Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation?. *Annual Review of Physiology,* 57, 19–42.

Hazel, J.R., Landrey, S.R. 1988. Timecourse of thermal acclimation in plasma membranes of trout kidney: 2. Molecular species composition. *American Journal of Physiology (Regulatory Integrated Comparative Physiology),* 255, 628–634.

Hazel, J.R., Williams, E.E. 1990. The role of alterations in membrane lipid-composition in enabling physiological adaptation of organisms to their physical environment. *Progress in Lipid Research,* 29, 167–227.

Heidelberg, J.F., Heidelberg, K.B., Colwell, R.R. 2002. Bacteria of the γ-subclass proteobacteria associated with zooplankton in Chesapeake Bay. *Applied and Environmental Microbiology,* 68, 5498–5507.

Henderson, R.J., Tocher, D.R. 1987. The lipid composition and biochemistry of freshwater fish. *Progress in Lipid Research,* 26, 281–347.

Hilditch, T.P., Williams, P.N. 1964. *The chemical condition of natural fat.* Chapman and Hall, London.

HMSO. 1994. *Nutritional aspects of cardiovascular disease: report on health and social subjects.* Committee of Medical Aspects of Food Policy, 46, Department of Health, HMSO, London, UK.

Hochachka, P.W., Somero, G.N. 2002. *Biochemical adaptation: mechanism and process in physiological evolution.* Oxford University Press, New York, USA, 560 pp.

Hofmann, G.E., Somero, G.N. 1996. Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination. *Marine Biology,* 126(1), 65–75.

Høi, L., Larsen, J.L., Dalsgaard, I., Dalsgaard, A. 1998. Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Applied and Environmental Microbiology,* 64(1), 7–13.

Hutchison, V.H., Dupré, R.K. 1992. Thermoregulation. *In:* Feder, M.E., Burgren, W. (Eds.), *Environmental Physiology of the Amphibia*. University of Chicago Press, Chicago, Illinois, USA, pp. 206–249.

ICMSF. 1986. Microorganisms in Foods 2. Sampling for Microbiological Analysis: Principles and Specific Applications (2nd ed.). *In: International Commission on Microbiological Specifications for Foods*. University of Toronto Press/Blackwell Scientific Publications, Toronto, 131 pp.

IPCC. 2013. *Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC),* Stocker, T.F., Qin, D., Plattner, G.K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp.

Jackson, K.L., Ogburn, D.M. 1999. *Review of depuration and its role in shellfish quality assurance (FRDC Project No. 96/355).* NSW Fisheries Final Report Series No. 13, Pyrmont, Australia, 77 pp.

Koff, R.S., Sear, H.S. 1967. Survival of viruses in steamed clams. *New England Journal of Medicine,* 276, 737–740.

Kregel, K.C. 2002. Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *Journal of Applied Physiology,* 92(5), 2177–2186.

Kural, A.G., Chen, H. 2008. Conditions for a 5-log reduction of *Vibrio vulnificus* in oysters through high hydrostatic pressure treatment. *International Journal of Food Microbiology,* 122, 180–187.

Kural, A.G., Shearer, A.E.H., Kingsley, D.H., Chen, H. 2008. Conditions for high pressure inactivation of *Vibrio parahaemolyticus* in oysters. *International Journal of Food Microbiology,* 127, 1–5.

Laing, I., Spencer, B.E. 2006. *Bivalve cultivation: criteria for selecting a site.* Cefas Science Series Technical Report No. 136, 34 pp.

Laruelle, F., Guillou, J., Paulet, Y.M. 1994. Reproductive pattern of the clams, *Ruditapes decussatus* and *R. philippinarum* on intertidal flats in Brittany. *Journal of the Marine Biological Association of the United Kingdom,* 74, 351–366.

Lee, R., Lovatelli, A., Ababouch, L. 2008. *Bivalve depuration: fundamental and practical aspects.* FAO Fisheries Technical Paper, No. 511, Rome, 161 pp.

Lesser, M.P. 2006. Oxidative stress inmarine environments: biochemistry and physiological ecology. *Annual Review of Physiology,* 68, 253–278.

Lhafi, S.K., Kühne, M. 2007. Occurrence of *Vibrio* spp. in blue mussels (*Mytilus edulis*) from the German Wadden Sea. *International Journal of Food Microbiology,* 116, 297–300.

Lockwood, B.L., Somero, G.N. 2011. Invasive and native blue mussels (genus *Mytilus*) on the California coast: the role of physiology in a biological invasion. *Journal of Experimental Marine Biology and Ecology,* 400, 167–174.

López-Joven, C., Ruiz-Zarzuela, I., de Blas, I., Furones, M.D., Roque, A. 2011. Persistence of sucrose fermenting and non-fermenting vibrios in tissues of Manila clam species, *Ruditapes philippinarum*, depurated in seawater at two different temperatures. *Food Microbiology,* 28, 951–956.

Maazouzi, C., Piscart, C., Legier, F., Hervant, F. 2011. Ecophysiological responses to temperature of the "killer shrimp" *Dikerogammarus villosus*: Is the invader really stronger than the native *Gammarus pulex*?. *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology,* 159, 268–274.

Martínez, O., Rodríguez-Calleja, J.M., Santos, J.A., Otero, A., García-López, M.L. 2009. Foodborne and indicator bacteria in farmed molluscan shellfish before and after depuration. *Journal of Food Protection,* 72, 1443–1449.

Martinez-Urtaza, J., Saco, M., de Novoa, J., Perez-Piñeiro, P., Peiteado, J., Lozano-Leon, A., Garcia-Martin, O. 2004. Influence of environmental factors and human activity on the presence of *Salmonella* serovars in a marine environment. *Applied and Environmental Microbiology,* 70, 2089–2097.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V. 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases,* 5, 607–625.

Miliotis, M. 2007. Role of microbial risk assessment in food safety. *SAMJ,* 97(11), 1211–1214.

Oliver, J.D. 2005. The viable but nonculturable state in bacteria. *Journal of Microbiology,* 43, 93–100.

Øverli, Ø., Sørensen, C., Nilsson, G.E. 2006. Behavioral indicators of stress-coping style in rainbow trout: do males and females react differently to novelty?. *Physiology & Behaviour,* 87, 506–512.

Pampanin, D.M., Volpato, E., Marangon, I., Nasci, C. 2005. Physiological measurements from native and transplanted mussel (*Mytilus galloprovincialis*) in the canals of Venice. Survival in air and condition index. *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology,* 140, 41–52.

Patrick, S., Faury, N., Goulletquer, P. 2006. Seasonal changes in carbohydrate metabolism and its relationship with summer mortality of Pacific oyster *Crassostrea gigas* (Thunberg) in Marennes-Oleron bay (France). *Aquaculture,* 252, 328–338.

Pernet, F., Tremblay, R., Comeau, L., Guderley, H. 2007. Temperature adaptation in two bivalve species from different thermal habitats: energetics and remodelling of membrane lipids. *Journal of Experimental Biology,* 210, 2999–3014.

Pörtner, H.O. 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology,* 132(4), 739–761.

Pörtner, H.O. 2010. Oxygen and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *Journal of Experimental Biology,* 213, 881–893.

Pörtner, H.O., Knust, R., 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science,* 315, 95–97.

Pörtner, H.O., Mark, F.C., Bock, C. 2004. Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques. *Respiratory, Physiology & Neurobiology,* 141(3), 243–260.

Prapaiwong, N., Wallace, R.K., Arias, C.R. 2009. Bacterial loads and microbial composition in high pressure treated oysters during storage. *International Journal of Food Microbiology,* 131, 145–150.

Qiu, J.-W., Xie, Z.-C., Wang, W.-X. 2005. Effects of calcium on the uptake and elimination of cadmium and zinc in Asiatic clams. *Archives of Environmental Contamination and Toxicology,* 48, 278–287.

Rejmánek, M. 1996. A theory of seed plant invasiveness: the first sketch. *Biological Conservation,* 78, 171–181.

Rejmánek, M. 2000. Invasive plants: approaches and predictions. *Austral Ecology,* 25, 497–506.

Richards, G.P. 1991. Shellfish depuration. *In:* Ward, D.R., Hackney, C.R. (Eds.), *Microbiology of marine food products*. Van Nostrand Reinhold, New York, pp. 395–428.

Ripabelli, G., Sammarco, M.L., Grasso, G.M., Fanelli, I., Caprioli, A., Luzzi, I. 1999. Occurrence of *Vibrio* and other pathogenic bacteria in *Mytilus galloprovincialis* (mussels) harvested from Adriatic Sea, Italy. *International Journal of Food Microbiology,* 49(1–2), 43–48.

Roderick, G.E., Schneider, K.R. 1994. Depuration and relaying of molluscan shellfish. *In:* Hackney, C.R., Person, M.D. (Eds.), *Environmental indicators and shellfish safety*. Chapman & Hall, New York, pp. 331–363.

Roesijadi, G., Unger, M.E. 1993. Cadmium uptake in gills of the mollusc *Crassostrea virginica* and inhibition by Ca channel blockers. *Aquatic Toxicology,* 24, 195–206.

Rosa, R., Seibel, B.A. 2008. Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. *Proceedings of the National Academy of Sciences of the United States of America,* 105, 20776–20780.

Rosa, R., Seibel, B.A. 2010. Voyage of the argonauts in the pelagic realm: physiological and behavioral ecology of the rare paper nautilus, *Argonauta nouryi*. *ICES Journal of Marine Science,* 67(7), 1494–1500.

Sala, O.E., Chapin, III F.S., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L.F., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D.M., Mooney, H.A., Oesterheld, M., Poff, N.L., Sykes, M.T., Walker, B.H., Walker, M., Wall, D.H. 2000. Global biodiversity scenarios for the year 2100. *Science,* 287, 1770–1774.

Sargent, J., Bell, G., McEvoy, L., Tocher, D., Estevez, A. 1999. Recent developments in the essential fatty acid nutrition of fish. *Aquaculture,* 177, 191–199.

Setti, I., Rodriguez-Castro, A., Pata, M.P., Cadarso-Suarez, C., Yacoubi, B., Bensmael, L., Moukrim, A., Martinez-Urtaza, J. 2009. Characteristics and dynamics of *Salmonella* contamination along the coast of Agadir, Morocco. *Applied and Environmental Microbiology,* 75(24), 7700–7709.

Simopoulos, A.P. 1991. Omega-3 fatty acids in health and disease and in growth and development. *American Journal of Clinical Nutrition,* 54, 438–463.

Sinensky, M. 1974. Homeoviscous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America,* 71(2), 522–525.

Skalli, J.H., Robin, N., Le Bayon, H., Delliou, L., Person-Le Ruyet, J. 2006. Impact of essential fatty acid deficiency and temperature on tissues' fatty acid composition of European sea bass (*Dicentrarchus labrax*). *Aquaculture,* 255, 223–232.

Somero, G.N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *Journal of Experimental Biology,* 213, 912–920.

Sorte, C.J.B., Williams, S.L., Zerebecki, R.A. 2010. Ocean warming increases threat of invasive species in a marine fouling community. *Ecology,* 91(8), 2198–2204.

Su, Y.-C., Liu, C. 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. *Food Microbiology,* 24, 549–558.

Surette, M.E., Gill, T.A., LeBlanc, P.J. 1988. Biochemical basis of postmortem nucleotide catabolism in cod (*Gadus morhua*) and its relationship to spoilage. *Journal of Agricultural and Food Chemistry,* 36, 19–22.

Suter, G. 2007. Freshwater bivalve ecotoxicology. *In:* Farris, J.L., Van Hassel, J.H. (Eds.), *Integrated Environmental Assessment and Management*, 3(4), 568–569.

Talmage, S.C., Gobler, C.J. 2011. Effects of elevated temperature and carbon dioxide on the growth and survival of larvae and juveniles of three species of Northwest Atlantic bivalves. *Plos One,* 6(10), e26941.

Thompson, F.L., Iida, T., Swings, J. 2003. *Biodiversity of Vibrios. Microbiology and Molecular Biology Reviews, 68, 403–431.*

Thorsen, W.A., Cope, W.G., Shea, D. 2007. Toxicokinetics of environmental contaminants in freshwater bivalves. *In:* Farris, J.L., Van Hassel, J.H. (Eds.), *Freshwater bivalves ecotoxicology*. CRC Press, Boca Raton, FL pp. 169–214.

Timofeyev, M.A., Shatilina, Z.M., Protopopova, M.V., Bedulina, D.S., Pavlichenko, V.V., Kolesnichenko, A.V., Steinberg, C.E.W. 2009. Thermal stress defense in freshwater amphipods from contrasting habitats with emphasis on small heat shock proteins (sHSPs). *Journal of Thermal Biology,* 34(6), 281–285.

Timoney, J.R., Abston, A. 1984. Accumulation and elimination of *Escherichia coli* and *Salmonella typhimurium* by hard clams in an in vitro system. *Applied and Environmental Microbiology,* 47, 986–988.

Tomanek, L., Somero, G.N. 1999. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *Journal of Experimental Biology,* 202, 2925–2936.

Truebano, M., Burns, G., Thorne, M.A.S., Hillyard, G., Peck, L.S., Skibinski, D.O.F., Clark, M.S. 2010. Transcriptional response to heat stress in the Antarctic bivalve *Laternula elliptica*. *Journal of Experimental Marine Biology and Ecology,* 391, 65–72.

Turner, J.W. 2010. *Environmental factors and reservoir shifts contribute to the seasonality of pathogenic Vibrio species.* PhD thesis, University of Georgia, Athens, 181 pp.

Turner, J.W., Good, B., Cole, D., Lipp, E.K. 2009. Plankton composition and environmental factors contribute to *Vibrio* seasonality. *The ISME Journal,* 3, 1082–1092.

Uzaki, N., Kai, M., Aoyama, H., Suzuki, T. 2003. Changes in mortality rate and glycogen content of the Manila clam *Ruditapes philippinarum* during the development of oxygen-deficient waters. *Fisheries Science,* 69, 936–943.

Van Dooremalen, C., Ellers, J. 2010. A moderate change in temperature induces changes in fatty acid composition of storage and membrane lipids in a soil arthropod. *Journal of Insect Physiology,* 56, 178–184.

Van Dooremalen, C., Koekkoek, J., Ellers, J. 2011. Temperature-induced plasticity in membrane and storage lipid composition: thermal reaction norms across five different temperatures. *Journal of Insect Physiology,* 57(2), 285-291.

Wang, Y., Evans, R.D. 1993. Influence of calcium concentrations on cadmium uptake by the freshwater mussel *Ellptio complanata*. *Canadian Journal of Fisheries and Aquatic Sciences,* 50(12), 2591–2596.

Williams, E., Somero, G. 1996. Seasonal-, tidal-cycle- and microhabitat-related variation in membrane order of phospholipid vesicles from gills of the intertidal mussel *Mytilus californianus*. *Jornal of Experimental Biology,* 199, 1587–1596.

Wittman, R.J., Flick, G.J. 1995. Microbial contamination of shellfish: prevalence, risk to human health, and control strategies. *Annual Review of Public Health,* 16, 123–140.

Wright, A.C., Hill, R.T., Johnson, J.A., Roghman, M.-C., Colwell, R.R., Morris Jr., J.G. 1996. Distribution of *Vibrio vulnificus* in the Chesapeake Bay. *Applied and Environmental Microbiology,* 62(2), 717–724.

Yang, Q. 2008. *Refrigerated seawater depuration for reducing Vibrio parahaemolyticus contamination in raw oysters (Crassostrea gigas).* MSc thesis, Oregon State University, 109 pp.

Zerebecki, R.A., Sorte, C.J.B. 2011. Temperature tolerance and stress proteins as mechanisms of invasive species success. *PLos One,* 6(4), e14806.

Annex

Eurostat codes

Description as in Eurostat	Eurostat code
Live flat oysters "*Ostrea* spp.", weighing ≤ 40 g each including shell	03071010
Oysters, live, fresh, chilled, frozen, dried, salted or in brine (excluding live flat oysters "*Ostrea* spp.", weighing ≤ 40 g each including shell)	03071090
Live flat oysters "*Ostrea*" weighing "including shell" ≤ 40 g	03071110
Oysters, even in shell, live, fresh or chilled (excluding live flat oysters "*Ostrea*" weighing "including shell" ≤ 40 g)	03071190
Oysters, smoked, even in shell, even cooked but not otherwise prepared	03071910
Oysters, even in shell, frozen, dried, salted or in brine (excluding smoked)	03071990
Live, fresh or chilled, scallops, including Queen scallops, of the genera *Pecten*, *Chlamys* or *Placopecten*, even in shell	03072100
Scallops, including Queen scallops, of the genera *Pecten*, *Chlamys* or *Placopecten*, smoked, even in shell, even cooked but not otherwise prepared	03072905
Coquilles Saint-Jacques "*Pecten maximus*", frozen, even in shell (excluding smoked)	03072910
Scallops, including Queen scallops, of the genera *Pecten*, *Chlamys* or *Placopecten*, frozen, dried, salted or in brine, even in shell (excluding smoked, and frozen coquilles Saint-Jacques "*Pecten maximus*")	03072990
Mussels "*Mytilus* spp.", live, fresh or chilled, with or without shell	03073110
Mussels "*Perna* spp.", live, fresh or chilled, with or without shell	03073190
Mussels "*Mytilus* spp., *Perna* spp.", smoked, even in shell, even cooked but not otherwise prepared	03073905
Mussels "*Mytilus* spp.", frozen, dried, salted or in brine, even in shell (excluding smoked)	03073910
Mussels "*Perna* spp.", frozen, dried, salted or in brine, even in shell (excluding smoked)	03073990
Live, fresh or chilled, even in shell, clams, cockles and ark shells "families Arcidae, Arcticidae, Cardiidae, Donacidae, Hiatellidae, Mactridae, Mesodesmatidae, Myidae, Semelidae, Solecurtidae, Solenidae, Tridacnidae and Veneridae"	03077100
Clams, cockles and ark shells "families Arcidae, Arcticidae, Cardiidae, Donacidae, Hiatellidae, Mactridae, Mesodesmatidae, Myidae, Semelidae, Solecurtidae, Solenidae, Tridacnidae and Veneridae", smoked, even in shell, even cooked but not otherwise prepared	03077910
Striped venus or other "Veneridae", even in shell, frozen	03077930
Frozen, dried, salted or in brine, even in shell, clams, cockles and ark shells "families Arcidae, Arcticidae, Cardiidae, Donacidae, Hiatellidae, Mactridae, Mesodesmatidae, Myidae, Semelidae, Solecurtidae, Solenidae, Tridacnidae and Veneridae" (excluding smoked)	03077990
Striped venus and other "Veneridae", even in shell, frozen (excluding smoked)	03079913